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GENETIC AND PHYSIOLOGICAL STUDIES OF BACILLUS ANTHRACIS RELATED TO DEVELOPMENT OF AN IMPROVED VACCINE

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

JULY 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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University of Massachusetts
Amherst, Massachusetts 01003

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The primary objective of the research is to gain information and to develop genetic systems that will contribute to development of an improved vaccine for anthrax. During the year represented by this report our research concentrated largely on (i) genetics of the toxin plasmid, pXO1, and the capsule plasmid, pXO2, of B. anthracis; (ii) further characterization of the Bacillus mating system encoded by fertility plasmids pXO11 and pXO12 of B. thuringiensis; and (iii) identification and characterization of additional fertility plasmids useful for transferring plasmids among Bacillus strains and species.

By plasmid transfer experiments we have shown that the different capsule phenotypes exhibited by variants of B. anthracis are plasmid-associated. Transcipients inheriting pXO2 displayed the capsule phenotype of the pXO2 donor strain.

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An important goal of our research is the development of a system for putting plasmid DNA manipulated *in vitro* back into nontransformable *Bacillus* species such as *B. anthracis*. We had hoped that one or more of the *B. thuringiensis* fertility plasmids would render transformable *B. subtilis* capable of transferring plasmids to *B. anthracis*. However, although *B. anthracis* strains harboring *B. thuringiensis* fertility plasmid pXO12, pXO13, pXO14, or pXO16 were effective in transferring the tetracycline resistance plasmid pBC16 to *B. subtilis*, the transcipients were not fertile because the fertility plasmids themselves were either not transferred or not maintained in *B. subtilis*. We have recently demonstrated that plasmid pLS20 of *B. subtilis* (natto) is capable of promoting the transfer of pBC16 from *B. subtilis* to a variety of *Bacillus* species including *B. anthracis*. Evidence suggests that a conjugation-like mechanism is involved. This is the first reported demonstration of a fertility plasmid in *Bacillus* species other than *B. thuringiensis*. Hopefully, this new system of conjugal transfer of plasmids will facilitate the introduction of plasmid DNA into nontransformable *B. anthracis* by using transformable and fertile *B. subtilis* strains as intermediates.

Evidence is accumulating that plasmid transfer in the *Bacillus* mating system encoded by *B. thuringiensis* fertility plasmids may involve cointegrate forms. Altered plasmids are frequently observed in transcipients. Some altered forms of pXO1 isolated from transcipients have been shown to have insertions of pXO12 fertility plasmid DNA. The function, if any, of the inserted DNA is being investigated.

Results of restriction endonuclease analysis of pXO1 and pXO2 from various strains of *B. anthracis* indicate that each of the plasmids is generally well conserved among the strains investigated.

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SUMMARY

This is a progress report (first annual report) of research being carried out on Bacillus anthracis under contract DAMD17-85-C-5212. Research on the contract which began August 1, 1985 is a continuation of research previously carried out under contract DAMD17-80-C-0099. During the year represented by this annual report our research concentrated largely on (i) genetics of the toxin plasmid, pXO1, and the capsule plasmid, pXO2, of B. anthracis; (ii) further characterization of the Bacillus mating system encoded by B. thuringiensis plasmids, pXO11 and pXO12; and (iii) identification and characterization of additional fertility plasmids useful for transferring plasmids among B. anthracis, B. cereus, B. thuringiensis, B. subtilis and other Bacillus species.

Results presented here demonstrate that the different capsule phenotypes of B. anthracis strains carrying pXO2 are plasmid-associated. Plasmid transfer experiments showed that noncapsulated mutants of $(pXO2)^+$ strains regained the ability to synthesize capsules when their plasmid was replaced with pXO2 from capsulated strains. The M strain of B. anthracis is unusual in that it does not require added CO_2 for synthesis of capsule. Plasmid transfer experiments demonstrated that the mutation engendering the M phenotype is plasmid-borne.

B. anthracis strains harboring B. thuringiensis fertility plasmid pXO12, pXO13, pXO14, or pXO16 were able to transfer the tetracycline resistance plasmid, pBC16, to B. subtilis. Unfortunately, the fertility plasmids themselves were either not transferred to B. subtilis or were not maintained in that organism. Therefore, these plasmids are not useful for constructing fertile strains of B. subtilis for the purpose of transferring plasmids from that transformable species to nontransformable B. anthracis. However, results of recent experiments demonstrate that pLS20, a 34-megadalton plasmid of B. subtilis (natto), is capable of promoting the transfer of plasmid pBC16 from B. subtilis to B. anthracis, B. cereus, B. megaterium, B. subtilis, and B. thuringiensis. Evidence for a plasmid-encoded conjugation-like method of plasmid transfer is presented. Hopefully, this new system of plasmid exchange will facilitate the introduction of plasmid DNA into nontransformable Bacillus species by use of transformable fertile B. subtilis strains as intermediates.

The mechanism of conjugal transfer of pXO1 mediated by B. thuringiensis fertility plasmids is being investigated. In initial matings the frequency of pXO1 transfer mediated by the fertility plasmid pXO12 was fairly low, but in subsequent matings using as donor a transcipient that inherited pXO1 and pXO12, the frequency of pXO1 transfer was increased significantly. Analysis of pXO1 from high frequency donors by restriction endonucleases and hybridization experiments showed that the plasmid had been altered by acquisition of some pXO12 DNA. The role, if any, of the acquired DNA in the increased transfer frequency has yet to be determined. Whether it is complementing transfer functions, or whether it enhances transfer by supplying a region of homology with pXO12 are areas we hope to explore.

Confirmation that the B. thuringiensis plasmids, pXO11, pXO12, pXO13, pXO14, and pXO16, are self-transmissible was obtained by the isolation of fertile B. anthracis and B. cereus transcipients that contain only pBC16 and one of these plasmids. We have not been able to isolate a strain carrying pXO15 as the sole B. thuringiensis plasmid and, therefore, have not obtained conclusive proof that it is self-transmissible. Differences in electrophoretic mobility of the B. thuringiensis fertility plasmids identified in our laboratory, indicated that they are not the same plasmid simply isolated from different subspecies of B. thuringiensis, and examination of the DNA restriction patterns generated by the different plasmids has led to the conclusion that they are not related solely on the basis of duplications or deletions of DNA at one site. When some of the plasmids were probed for DNA homology, considerable homology was found, but the existence of nonhomologous DNA restriction fragments for each plasmid confirmed their nonidentity. Their homology might have a basis in the presence of common gene sequences that encode their conjugation and transmission functions, but this remains to be determined.

Restriction analysis of three independent transfer defective deletion derivatives of pXO12 indicate that gene(s) responsible for conjugal transfer ability are located on a 70-kilobase PstI fragment of pXO12. A deletion mutant of pXO11 has been shown to be a more effective fertility plasmid than its parent, wild-type pXO11. Donors carrying the mutant plasmid transferred pBC16 at a 10^2- to 10^3-fold higher frequency than donors carrying wild-type pXC11. One possible explanation for this phenomenon is that the deleted DNA sequences code for a repressor or a repressor binding site involved in

regulation of pXO11 transfer function(s). Thus, pXO11 may normally exist in a repressed state and loss of gene(s) involved in regulation may generate plasmids that are de-repressed with respect to fertility.

Plasmids pXO1 and pXO2 from various strains of B. anthracis were examined by restriction endonuclease analysis. The results suggest that each of the plasmids is generally well conserved among the various strains examined, with only small differences revealed by digestion with EcoRI and PstI restriction enzymes.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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ANNUAL PROGRESS REPORT

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During the year represented by this annual report our research concentrated largely on (i) genetics of the toxin plasmid, pXO1, and the capsule plasmid, pXO2, of B. anthracis; (ii) further characterization of the Bacillus mating system encoded by B. thuringiensis plasmids pXO11 and pXO12; and (iii) identification and characterization of additional Bacillus fertility plasmids useful for transferring plasmids among B. anthracis, B. cereus, B. subtilis, and other Bacillus species. In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. Table 1 lists the strains and mutants referred to in this report.

Media. For convenience to the reader, compositions of the various culture media referred to in this report are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

$NBYCO_3$ agar: NBY agar with 7 g of $NaHCO_3$.

Phage assay (PA) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4.7H_2O$, 0.2 g; $MnSO_4.H_2O$, 0.05 g; $CaCl_2.2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

$PACO_3$ agar: PA agar with 7 g of $NaHCO_3$.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

LG broth: L broth with 1 gm of glucose.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

SG medium: This medium for polyglutamate production by B. subtilis (natto) was described by Hara et al. (6). It contained L-glutamic acid (monosodium salt), 15 g; KH_2PO_4, 2.7 g; $Na_2HPO_4.12H_2O$, 4.2 g; NaCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; sucrose, 50 g; and biotin, 1 mg. The pH was adjusted to 6.4.

DM3 medium: This medium for regenerating protoplasts in transformation experiments was prepared as described by Chang and Cohen (4).

CA-agarose medium: CA-agarose medium for the detection of colonies producing protective antigen were prepared as follows: 0.75 g of agarose was added to 100 ml of CA broth [prepared as described by Thorne and Belton (27)] and the mixture was steamed until the agarose was dissolved. When the medium cooled to about 50°C, 1 ml of 20% glucose, 8 ml of 9% $NaHCO_3$, 6 ml of goat antiserum to B. anthracis, and 10 ml of horse serum were added. The medium was dispensed in petri plates (13 ml per plate) and the plates were left with their lids ajar while the agarose solidified. The plates were usable after 1 hr.

Antisera. All B. anthracis antisera were kindly supplied by personnel of USAMRIID.

Propagation and assay of bacteriophage CP-51 and CP-54. The methods described previously (19, 22, 23) were followed. The indicator for routine

assay of these phages was B. cereus 569.

Test for capsule production. The ability of B. anthracis and B. cereus to produce capsules was determined by growing cells on PA agar incubated in air, or on $PACO_3$ agar incubated in 20% CO_2. Plates were incubated at 37C for 24 to 48 h.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (10). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10 µg/ml). With some strains better results were obtained when 0.5% (w/v) glycerol was included in the BHI broth to prevent sporulation. Best results with strains of B. anthracis were obtained when the BHI broth was supplemented with 10% (v/v) horse serum. The inoculum for each flask was a loop of growth from an L agar plate which had been streaked with a loop of spores and incubated at 37°C for 16 to 24 hours. Cultures were incubated at 37°C on a rotary shaker (100 to 160 rpm) for 13 to 16 hours. Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 0.002 M EDTA (tetrasodium salt, Sigma), 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 gm of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. Five-tenths ml of Pronase (Calbiochem-Behring Corp., La Jolla, CA) solution (2 mg per ml in 2 M Tris at pH 7.0) was added, and the tubes were mixed as above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C and the aqueous phase was removed for electrophoresis.

This procedure as described above was used for B. anthracis, B. cereus, and B. thuringiensis. The procedure was modified slightly for B. subtilis and B. licheniformis. Cultures were grown in BHI broth supplemented with 0.1% glycerol for 16 hours. After cell pellets were suspended in 2 ml of E buffer, lysozyme was added to give a final concentration of 2 mg per ml, and suspensions were incubated at 37°C for 45 to 60 minutes. Two ml of lysis buffer as described above were added and tubes were inverted 20 times. After the suspensions were incubated 30 minutes at 60°C they were cooled on ice and 0.5 ml of 2M Tris-OH

was added. The tubes were inverted 20 times, and following the addition of 6 ml of cold phenol-chloroform mixture to each tube, they were inverted 20 times again. Finally the tubes were centrifuged at 10,000 rpm for 10 minutes and the aqueous layer was withdrawn.

For electrophoresis of plasmid DNA, extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40 µl) were applied to horizontal 0.7% agarose (Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 0.089 M boric acid, 0.0025 M EDTA, pH 8.2 to 8.3). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (1 µg/ml) in Tris-borate buffer).

Method for isolating plasmid DNA suitable for restriction analysis. The above procedure for extracting plasmid DNA has been modified in such a way that very little or no chromosomal DNA is present in the preparations. At least there is not enough chromosomal DNA present to interfere with restriction analysis of plasmids. The procedure is simple and does not involve density gradient centrifugation. By removing unlysed cells from lysates after incubating cells in lysis buffer at 60°C and chilling the lysates before extracting them with phenol-chloroform, we obtain plasmid DNA that is essentially free of chromosomal DNA. A description of our entire modified procedure is included here for the convenience of those who might wish to use it.

The modified procedure is the same as the procedure described above through the lysis step at 60°C. At that point cell debris and unlysed cells were removed by centrifugation at 10,000 rpm for 15 minutes at 5°C. The supernatant fluid was decanted and placed in an ice bath. Ice cold 2 M Tris (0.5 ml, pH 7.0) was added to neutralize the lysate and mixing was accomplished by inverting the tubes 20 times. The lysate was extracted with 6 ml of cold phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The emulsions were separated by centrifugation at 10,000 rpm for 10 minutes at 5°C, and the aqueous phase was removed. One-half volume of 7.5 M ammonium acetate was added, and the plasmid DNA was precipitated by adding twice the final volume of ice cold 95% ethanol. The tubes were held on ice for 15 minutes and then centrifuged at 10,000 rpm for 30 to 60 minutes at 5°C. The ethanol was decanted, 5 ml of cold 70% ethanol was added to each tube which was then mixed gently on a vortex mixer, and the DNA was collected by centrifugation at 10,000

rpm for 15 minutes at 5°C. After the ethanol was decanted, the tubes were inverted over paper towels for 10 minutes and then placed in a vacuum desiccator for at least 2 hours to dry the DNA thoroughly. The DNA was dissolved in 0.1 to 0.5 ml of TES (0.05 M Tris-OH, 0.05 M NaCl, 0.005 M EDTA, pH 8.0) containing 50 µg of RNase per ml.

If larger preparations of plasmid DNA are desired, the above procedure can be scaled up successfully.

Plasmid DNA for nick translation was further purified by cesium chloride density gradient centrifugation.

Restriction endonuclease digestions. Restriction endonuclease digestions were carried out under conditions recommended by the supplier of the enzymes. Usually 10 to 20 µl of DNA (1.0 to 1.5 µg) in TES (pH 8.0) was added to 5 to 10 units of enzyme in a 1.5 ml Eppendorf tube. Appropriate amounts of distilled water and 10X buffer were added to give a total volume of 100 µl. Reaction mixtures were incubated in a 37°C water bath for 2 to 15 h. Digests were heated at 65°C for 10 minutes to stop reactions and then resolved on agarose gels.

Transduction of pXO2. Bacteriophage CP-51ts45 was propagated on B. anthracis and assayed on B. cereus 569. Recipient cells for transduction were grown in 250-ml flasks containing 25 ml of L broth (for B. cereus) or BHI broth with 0.5% glycerol (for B. anthracis) and incubated at 37°C on a rotary shaker at 250 rpm. Cells from a 10% (vol/vol) transfer of a 16-h culture were grown for 5 h. Cells (0.1 ml containing approximately 10^8 CFU) and phage (0.1 ml containing approximately 5×10^9 PFU) were spread together on $NBYCO_3$ or $PACO_3$ agar. Plates were incubated at 37°C in 20% CO_2. After 3 h, 0.1 ml of phage CP-54 (3×10^9 PFU) was spread on the transduction plates to lyse noncapsulated cells and to allow the selection of capsulated transductants. Incubation in CO_2 was continued for 36 to 48 h.

Protoplast transformation. B. subtilis (natto) was transformed by the method of Chang and Cohen (4). Plasmid pBC16 DNA for transformation was obtained from B. anthracis UM23C1-2 tr43K-5(pBC16) by the modified method of Kado and Liu described above.

Procedures used in mating experiments:

(1) Matings in broth: Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in

BHI broth, yielding 10^6 to 10^7 cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transcipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transcipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml. If the recipients were streptomycin-sensitive, tetracycline-resistant transcipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transcipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transcipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transcipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10 µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transcipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

(2) Matings on membranes: The procedures used in carrying out matings on membranes are given in the appropriate sections of the text.

Screening colonies for protective antigen production. Colonies were picked to plates of CA-agarose medium and incubated at 37°C in 20% CO_2 for about 16 h. A zone of precipitate formed around colonies that produced protective antigen.

RESULTS AND DISCUSSION

I. Plasmid-related differences in capsule production by Bacillus anthracis

Results presented below demonstrate that the different capsule phenotypes

of pXO2$^+$ strains are plasmid-associated. Plasmid transfer experiments have shown that noncapsulated cured isolates express the capsule phenotype of the donor strain upon acquisition of pXO2. Therefore, the mutations which engender the rough phenotype of 6602 R4 and 6602 R5 and relieve the CO_2 requirement for capsule formation in strain M are plasmid-associated. 6602 R4 and R5 probably carry point mutations on the plasmid which render these strains Cap$^-$, since Cap^{+c} revertants are readily obtainable, and the plasmid restriction patterns do not differ from those of pXO2 from strain 6602. (Cap^{+c} is the phenotypic designation for strains that require CO_2 for capsule synthesis; Cap$_{+a}$ is the phenotypic designation for strains that synthesize capsules in air without added CO_2. Plasmid pXO2 from strain M is slightly altered as is evident from restriction analysis. However, this alteration is not associated with the ability to produce capsules in air, since variants of M which require CO_2 for capsule syrthesis and the Cap^{+c} strain NHΔ1 contain plasmids with the same alteration.

Although pXO2 has been demonstrated to be involved in the formation of capsule by B. anthracis, little is known regarding possible regulatory genes which may be present on the plasmid or on the B. anthracis chromosome. Further investigation of pXO2 is of interest, especially with regard to the CO_2 requirement for capsule synthesis.

Capsule phenotypes. B. anthracis plasmid pXO2 carries information for synthesis of a D-glutamyl polypeptide capsule. Previous work demonstrated that strains cured of this plasmid were noncapsulated and capsule synthesis was restored upon reintroduction of pXO2 by mating or transduction (5). B. anthracis strains harboring pXO2 can be divided into 3 groups with respect to capsule phenotype. Strains AmesΔ1, NHΔ1, and the ATCC Pasteur vaccine strains, 4229 and 6602, produce capsule only when grown on media containing serum or bicarbonate and incubated in a CO_2-rich atmosphere. Rough variants of 6602, 6602 R4 and 6602 R5, are noncapsulated under all growth conditions (Cap$^-$), yet retain pXO2. Spontaneous Cap$^+$ isolates arising from 6602 R4 and 6602 R5 require CO_2 for capsule production. Strain M does not exhibit a CO_2 requirement for capsule production. This strain is capsulated when grown in air as well as in CO_2. Colonies of 6602 and M grown in air and in 20% CO_2 are shown in Fig. 1.

Plasmid restriction analysis. Plasmid DNA preparations were digested with various endonucleases and the restriction digests were compared on 0.6% agarose

gels. EcoRI and HindIII/PstI digestions of pXO2 DNA from strains representing each phenotype (6602 Cap^{+c}, 6602 R4 Cap^{-}, M UM2 Cap^{+a}, and M UM3 Cap^{+c}) were compared. No differences in plasmid size (57 Mdal) or complement of restriction fragments were found, except with pXO2 from strain M. In this strain the plasmid appears to contain an additional 50 to 60 bp of DNA as indicated by the reduced mobility of the restriction fragments shown. The same alteration was seen when pXO2 DNA from the Cap^{+c} variant, M UM3, was digested. A survey of the restriction patterns of pXO2 from the vaccine resistant strains, AmesΔ1 and NHΔ1, revealed that the plasmid from strain NHΔ1 had the same alteration as that observed for the plasmid from strain M (see below).

Plasmid transfer experiments. To determine whether the gene(s) associated with the three capsule phenotypes (Cap^{-}, Cap^{+c}, and Cap^{+a}) were carried on the capsule plasmid or on the chromosome, pXO2 was transferred by CP-51-mediated transduction or by mating. Table 2 shows the results of transduction of pXO2 from strain 6602 into B. cereus and $pXO2^{-}$ strains of B. anthracis. Bacteriophage CP-51ts45, propagated on B. anthracis 6602, was used to transduce pXO2 into B. anthracis Weybridge A UM23C1, B. cereus 569 UM20-1, and B. anthracis M UM4. Phage CP-54, which lyses noncapsulated cells, allowed selection of Cap^{+} transductants. The presence of pXO2 in Cap^{+} transductants was confirmed by plasmid analysis. All $pXO2^{+}$ transductants exhibited the capsule phenotype of the 6602 donor; CO_2 was required for capsule synthesis.

If pXO2 from 6602 R4 or 6502 R5 were transduced to a formerly Cap^{+} strain subsequently cured of pXO2, presumably the transductants carrying pXO2 would be Cap^{-} if the mutation engendering the rough phenotype of 6602 R4 or R5 is carried on the plasmid. However, such a transduction experiment is not feasible since we know of no way to select Cap^{-} transductants. Therefore an experiment was done to test whether cured derivatives of 6602 R4 and R5 would exhibit the Cap^{+} phenotype when infected with pXO2 from a Cap^{+} strain. The recombinant plasmid, pXO12::pXO2, was introduced into cured strains by mating. Plasmid pXO12::pXO2 contains the B. thuringiensis fertility and parasporal crystal genes of pXO12 and the capsule genes of pXO2 from B. anthracis strain 6602 (Cap^{+c}).

B. cereus 569 UM20-1 tr60G-10(pXO12::pXO2, pBC16) Ant^{-} was mated with the cured B. anthracis strains 6602 R4 C1 and 6602 R5 C1. Tc^{r} Ant^{+} transcipients were screened by phase microscopy for parasporal crystals and subsequently examined for the ability to produce capsules. The results of these matings are shown in Table 3. Transfer of pBC16 to B. anthracis 6602 R4 C1 occurred at a

frequency of 1.6×10^{-3}. Two of four transcipients examined acquired pXO12::pXO2. Plasmid pBC16 was transferred to B. anthracis 6602 R5 C1 at a frequency of 3.1×10^{-4} and two of nine transcipients examined contained the recombinant plasmid. Thus, when cured isolates of 6602 R4 and R5 inherited pXO12::pXO2, they produced capsules in the presence of CO_2. The presence of pXO2 in the Cap^+ transcipients was confirmed by plasmid analysis. This suggests very strongly that the mutations which rendered 6602 R4 and R5 Cap^- were plasmid-borne.

To corroborate transduction results cited above which suggest that the capsule phenotype of strain M is plasmid-associated, the capsule genes from strain M (Cap^{+a}) were transferred by mating to a B. anthracis strain previously cured of pXO2. A fertile pXO2.1 donor (pXO2.1 designates the capsule plasmid from strain M) was constructed by mating B. thuringiensis 4059 UM1(pXO14, pBC16) with B. anthracis M UM2(pXO2.1). Tc^r transcipients were obtained which had acquired the fertility plasmid pXO14 in addition to pBC16. One of these transcipients, M UM2 tr24K-5, was mated with Weybridge A UM23 C1-2, as shown in Table 3. Three Tc^r Cap^+ transcipients were obtained in 20% CO_2 without CP-54 selection. Initially, all three transcipients, UM23 C1-2 tr30K-4, tr30K-5, and tr30K-6, appeared rough when grown in air. However, when overnight cultures were spread onto PA agar with CP-54, mucoid colonies were found distributed over the lysed rough background. These mucoid colonies continued to produce capsules when streaked to PA agar and incubated in air.

Deletion derivatives of pXO2.1. Matings in which the capsule plasmid from strain M was transferred to cured B. anthracis strains resulted in the generation of altered forms of pXO2.1 and pXO14. When B. thuringiensis 4059 UM1(pXO14, pBC16) was mated with B. anthracis M UM2, as described above, all Tc^r transcipients tested acquired pXO14 in addition to pBC16. One of these transcipients, tr24K-5, which was Spo^-, was transferred on NBY slants several times before cells were frozen in 25% glycerol. When this strain was used as a donor to B. anthracis UM23 C1-2, three Cap^{+a} transcipients were obtained without CP-54 selection in 20% CO_2. Plasmid analyses revealed that each of the transcipients, B. anthracis UM23C1-2 tr30K-4, tr30K-5, and tr30K-6, contained pBC16 and a large plasmid which could not be identified as pXO2.1 or pXO14. (pXO2 and pXO14 migrate very close to each other in 0.6% agarose gels). Two of the transcipients, tr30K-4 and tr30K-5, also carried a plasmid which appeared to be a deletion derivative of either pXO2.1 or pXO14. When the donor strain, M

UM2 tr24K-5 was analyzed again for plasmids, no large plasmids were apparent in the plasmid profile, but a smaller plasmid which migrated at the same rate as the small plasmid seen in tr30K-4 was present.

The transcipients were tested for fertility by mating with B. cereus 569 UM20-1. All three transferred pBC16 to B. cereus. Subsequent matings with B. anthracis UM23C1-2 and B. cereus 569 UM20-1 were performed in an effort to isolate the large and small plasmids of B. anthracis UM23 C1-2 tr30K-4, tr30K-5, and tr30K-6. Results of these matings suggested that the large plasmids in tr30K-4 and tr30K-6 are recombinants of plasmids pXO14 and pXO2.1. However, tr30K-5 was found to contain pXO14, pBC16, and a deletion derivative of pXO2.1. This deletion derivative, designated pXO2.1Δ1, has been isolated in B. anthracis UM23 C1-2 tr43K-3 C1. Preliminary results of restriction analysis indicate an approximate size of 24 Mdal for pXO2.1Δ1. This is less than one half of the size of unaltered pXO2.1 (57 Mdal). These results indicate that less than one-half of the DNA of pXO2.1 is required for capsule synthesis.

Selection of M-type strains. Unlike most Cap^+ B. anthracis strains, B. anthracis M is able to synthesize capsular material in the absence of added bicarbonate or CO_2. Although strains having the "M" or Cap^{a+} phenotype have been reported only rarely, phage selection can be used to isolate Cap^{+a} variants from a population of strain 6602 cells. When approximately 10^8 log phase cells were spread with the same number of CP-54 PFU on rich medium and incubated in air, Cap^{+a} isolates were obtained at a frequency of approximately 1×10^{-8}. Thus, with phage selection, "M-like" variants can be isolated from the prototypical $pXO2^+$ strain, 6602.

Strain M is relatively unstable with regard to capsule formation and plasmid content. Rough variants, i.e., ones that are Cap^- in air occur at high frequencies. Such variants were found to be of two types; one type had lost pXO2 and the other type retained the plasmid. At least some of the latter type are mutants which require CO_2 for capsule synthesis as evidenced by the fact that variants which require CO_2 for capsule synthesis can be isolated as spontaneously arising rough colonies in air (figure 1).

Experiments were performed in which cells from broth cultures of strains carrying pXO2 or pXO2.1 were challenged with phage CP-54 on PA agar incubated in air. Overnight cultures of the $pXO2^+$ strains 6602, 6602 R4, 6602 R5, 4229, and M UM22; the $pXO2.1^+$ strain M UM2; and the transcipient, M23 C1-2 tr30K-4, which carries pXO2.1 derived from strain M UM2, were plated in duplicate on PA agar.

The plates were incubated in air. After 2 h, 4×10^8 PFU of CP-54 were spread onto one set of plates. Duplicate plates were replicated onto PA agar which had been spread with the same number of PFU. Cap^{+a} colonies arose on a background of lysed cells after 24 to 48 h. The results shown in Table 4 indicate clearly that Cap^{+a} variants were present at a higher frequency among strains which contained capsule genes derived from strain M. B. anthracis UM23 C1-2 tr30K-4 was like strain M UM2 in that numerous Cap^{+a} colonies were obtained. The M transductant, M UM22, which contains the capsule plasmid from strain 6602, yielded only 2 independent Cap^{+a} isolates when tested. These observations strongly support the idea that the capsule phenotype of the M strain is plasmid-related.

II. Participation of B. subtilis in the mating system directed by fertility plasmids of B. thuringiensis

We reported previously (26) that B. subtilis could act as a recipient in matings with B. anthracis(pXO12, pBC16) as the donor. This has been confirmed in many experiments. However, we also reported that pXO12 could be transferred to B. subtilis and that B. subtilis transcipients inheriting pXO12 could serve as donors of pBC16 in matings with B. anthracis recipients. We have not been able to repeat this latter observation routinely, and we believe the problem lies in the instability of large plasmids such as pXO12 in B. subtilis. In addition to confirming that B. anthracis harboring pXO12 can transfer pBC16 to B. subtilis, recent experiments have shown that the fertility plasmids pXO13, pXO14, and pXO16 derived from B. thuringiensis are also effective in directing the transfer of pBC16 from B. anthracis to B. subtilis. Results of pertinent experiments are reported here.

Filter mating procedures for transfer of pBC16. B. subtilis was an ineffective recipient when incubated with potential donors in broth according to our standard procedure described above. However, B. subtilis could serve as an effective recipient when matings were carried out on membrane filters. Two procedures were used. For both procedures 6 ml of BHI broth in a 20-mm cotton-plugged tube was inoculated with 0.1 ml of a spore suspension of donor or recipient and incubated at 30°C on a slow shaker (about 80 excursions/minute) for approximately 16 hours. A 5% (v/v) transfer was made to fresh broth and incubation was continued for 5 hours. In one procedure 1 ml of donor and 1 ml

of recipient cells were mixed and impinged on a 25-mm Millipore HA membrane by applying mild suction, and the membrane was placed on non-selective medium to allow mating to occur. BHI agar was used if B. anthracis, B. cereus, or B. thuringiensis was the recipient. For all other recipients, PA agar was the medium of choice. After this period of incubation cells were collected from the membrane in 1 ml of 1% peptone and appropriate dilutions were plated on selective medium containing tetracycline to select transcipients that inherited pBC16. The selective plates were incubated at 30C for 1-2 days. In the other procedure equal volumes of donor and recipient cells were mixed together and 0.2 ml was spread on a 47-mm Millipore HA membrane. The membrane was incubated on nonselective medium for 4 hours and then it was transferred to selective medium containing tetracycline. Incubation was continued until colonies growing on the membrane could be scored. The advantage of this second method is that each colony represents an independent mating event.

Ability of various Bacillus strains carrying pXO11 and/or pXO12 to transfer pBC16 to B. subtilis. A survey of various Bacillus donors was conducted to determine whether B. subtilis could participate as a recipient in mating. Initially, broth matings were performed as described under Materials and Methods between B. cereus, B. thuringiensis, and B. anthracis donors harboring pXO11 and/or pXO12 and B. subtilis 168 trpC2. Selection was for B. subtilis transcipients that inherited pBC16. Such matings carried out in broth were generally unsuccessful, although occasionally a few transcipients (2 to 8 per ml) were isolated from mixtures in which B. thuringiensis harboring pXO12 was the donor. Therefore, filter matings by the impingement method described above were tried with greater success. Results are shown in Table 5.

B. thuringiensis and B. cereus donors harboring pXO11 or pXO12 were ineffective in promoting the transfer of pBC16 to B. subtilis 168. However, B. anthracis UM23 C2 tr237-10, containing pXO12 and pBC16, was an effective donor. Plasmid analysis of representative Tc^r B. subtilis transcipients confirmed the presence of pBC16, but pXO12 was never observed in such transcipients. (In other experiments B. anthracis strains carrying pXO11 were ineffective in transferring pBC16 to B. subtilis).

Results of the following experiments provided strong evidence that transfer of pBC16 from B. anthracis to B. subtilis 168 occurred by a conjugation-like process rather than spontaneous transformation. pBC16 was introduced into B. anthracis UM23C1 by transduction and the transductant, which carried no

fertility plasmids was tested as a donor to B. subtilis 168 in a filter mating. Selection was made for B. subtilis cells which had acquired pBC16; no Tc^r B. subtilis colonies were found. The effect of adding DNAse to mating mixtures prepared with a fertile donor was also tested. Fifty µg of DNAse per ml was added to the donor and recipient cultures 10 minutes prior to mixing and impinging on the filter and, in addition, DNAse was added to the nonselective medium. The presence of DNAse did not have any significant effect on the number of tetracycline-resistant B. subtilis transcipients produced.

Time and frequency of pBC16 transfer from B. anthracis to B. subtilis. Previous results from our laboratory had shown that for plasmid transfer to occur among strains of B. thuringiensis, B. anthracis and B. cereus a period for donor and recipient cells to grow together was required. The greatest number of transfer events occurred between 2 and 6 hours. It was of interest to determine whether pBC16 transfer from B. anthracis to B. subtilis occurred in a similar manner with respect to time.

Matings were performed in which the pXO12-containing B. anthracis donor UM23 C2 tr237-10 and B. subtilis 168 were grown and mixed as described above and impinged on a series of HA membranes. The membranes were placed on non-selective medium for various periods of time, after which the cells were plated on selective medium containing tetracycline. The results, shown in Table 6, are similar to those obtained previously with other strains in mating experiments carried out in broth. When the cells were plated on selective medium immediately after mixing, no tetracycline-resistant transcipients were observed. After two hours of mixed incubation, there was a significant number of Tc^r transcipients (3.6×10^2 per membrane), and the number increased rapidly during the next 4 hours to 1.1×10^4. During the period between 6 hours and 20 hours of mixed growth, there was a further increase of only 2-fold in the number of transcipients.

Further experiments were carried out by the second procedure described above which allows scoring of independent mating events. Membranes were spread with 0.2 ml of mixed culture and incubated on nonselective medium 4 hours before being transferred to selective medium. With this procedure 1.3×10^3 Tc^r colonies of B. subtilis transcipients were obtained per ml of mixed culture. These results demonstrate that pXO12 is quite effective in promoting the transfer of pBC16 from B. anthracis to B. subtilis.

Transfer of pBC16 to B. subtilis from B. anthracis donors carrying pXO13,

pXO14, or pXO16. It was of interest to determine whether other fertility plasmids originating in B. thuringiensis could also effect the transfer of pBC16 to B. subtilis. B. anthracis donors containing pBC16 and one of the fertility plasmids pXO13, pXO14, or pXO16 were tested for their ability to transfer pBC16 to B. subtilis in filter matings carried out by the impingement procedure described above. B. subtilis strains which were restriction- and modification-negative were used in these tests, and donors carrying pXO11 or pXO12 were included for comparison. The results of these matings are shown in Table 7.

Each of the newly-characterized fertility plasmids, pXO13, pXO14, and pXO16 was effective in promoting the transfer of pBC16 from B. anthracis to B. subtilis. pXO13 appeared to be the most effective of the three. The pXO11-containing donor was ineffective with the restriction-minus strains as it had been in previous experiments with B. subtilis 168 as the recipient. Numerous transcipients were isolated from each mating and analyzed for their plasmid content to determine whether any had acquired a fertility plasmid from the donor. As with all Tc^r B. subtilis transcipients analyzed previously, pBC16 was the only plasmid found.

Matings with B. licheniformis as a recipient. Because B. licheniformis is highly transformable, we tested it as a recipient in matings with B. anthracis. Since we were unable to isolate fertile B. subtilis transcipients, we hoped that pXO12 might be transferred to and maintained in B. licheniformis and thus provide a route to getting in vitro-manipulated plasmids back into B. anthracis. Therefore, the B. anthracis donor UM23 C2 tr237-10 was tested in filter matings with B. licheniformis strains 9945A UM109 and 11946 UM13. No Tc^r transcipients were obtained with strain 9945A UM109. However, strain 11946 UM13 was an adequate recipient, yielding approximately 4.0×10^2 Tc^r transcipients per filter. All of 64 transcipients analyzed for plasmid content had acquired pBC16, but none had acquired pXO12.

III. Demonstration of a fertility plasmid in Bacillus subtilis (natto)

B. subtilis (natto) produces "natto", a traditional fermentation food in Japan. Natto consists of polysaccharide and polyglutamate (PGA). The polyglutamate is mainly γ-PGA, containing D- and L-glutamate in varying

proportions (6). Small plasmids (about 3.6 Mdal) have been associated with PGA production in various strains of natto (6, 7, 9).

B. subtilis (natto) plasmids have been classified into four types, on the basis of molecular weight and restriction endonuclease analysis. In 10 of 15 strains screened by Tanaka and Koshikawa (21), one or more plasmids of 3.6, 4.0, 34, and 46 Mdal have been found. Strain 3335 harbors a 34 Mdal plasmid, pLS20, and a 3.6 Mdal plasmid, pLS19. Plasmid pLS19 is homologous to the polyglutamate plasmid pUH1 of the Asahikawa strain of B. subtilis (natto) (8, 9), and thus carries genes necessary for production of PGA. The function of the large plasmid, pLS20, in strain 3335, and plasmids similar in size to pLS20 found in other B. subtilis (natto) strains, has not been determined.

Plasmid pLS20 and other type 3 plasmids of the same size are present together with type 1 (3.6 Mdal) or type 2 (4.0 Mdal) plasmids in B. subtilis (natto) strains. Tanaka and Koshikawa (21) suggested, but did not demonstrate, that type 3 plasmids may be transferred by some unknown mechanism into cells in which either a type 1 or type 2 plasmid is present. B. subtilis (natto) strains are isolated from a vegetable cheese prepared by fermentation of boiled soybeans. Thus, during fermentation cells have the opportunity for contact. These considerations, in conjunction with our current work on B. thuringiensis fertility plasmids, prompted our investigation of possible fertility plasmids in B. subtilis (natto).

Results of experiments presented below demonstrate that the 34 Mdal plasmid of B. subtilis (natto) 3335, designated pLS20, is capable of promoting the transfer of plasmid pBC16 to B. anthracis, B. cereus, B. megaterium, B. subtilis, and B. thuringiensis. Evidence for a plasmid-encoded conjugation-like method of genetic exchange includes: (1) pLS20$^+$ strains, but not pLS20$^-$ strains, functioned as donors of pBC16, (2) incubation of donor and recipient cultures in the presence of DNase, followed by mating in the presence of DNase, did not affect plasmid transfer, and (3) cell-free filtrates of donor cultures did not convert recipient cells to Tc^r. Hopefully, this new system of plasmid exchange will facilitate the introduction of plasmid DNA into nontransformable Bacillus species by use of the transformable fertile B. subtilis (natto) strain as an intermediate.

Plasmid content of B. subtilis (natto) strains. B. subtilis (natto) 3335, which is Pga$^+$, contains the 3.6 Mdal PGA plasmid pLS19 and the 34 Mdal plasmid pLS20. The spontaneously cured isolate 3335 UM4 carries only pLS20 and is Pga$^-$.

The tetracycline resistance plasmid pBC16 was introduced into this strain by protoplast transformation and by mating. A rifampicin resistant mutant, 3335 UM5 was transformed with pBC16 to yield strain 3335 UM8. Two other pBC16$^+$ pLS20$^+$ strains were generated by mating strain 3335 UM4 with Weybridge A UM23 C1-2 tr43K-1(pXO14, pXO2.1Δ1, pBC16), yielding UM4 tr55K-1, and with Weybridge A UM23 C2 tr60B-1(pXO12, pBC16), yielding UM4 tr537B-15. One Tc^r transcipient, UM4 tr537B-16, lost pLS20 upon acquisition of pBC16. A plasmid negative B. subtilis (natto) strain, tr537B-16 C1, was derived by curing tr537B-16 of pBC16 by growing cells at 43°C.

Plasmid transfer from B. subtilis (natto) to B. anthracis. Membrane matings employing B. subtilis (natto) were performed as follows. Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated on a shaker (80 rpm) at 30°C. Donor and recipient cells were grown separately for 5 h from 5% (v/v) transfers of 14- to 16-h cultures. One ml of donor cells and 1 ml of recipient cells were mixed and 0.1 ml of the mixture was spread on a 47-mm Millipore DA membrane placed on LG agar. To determine the number of donor and recipient cells per membrane, the mixture was diluted and plated on the appropriate selective media. Control mixtures contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Plates were incubated at 30C for 5h to allow mating and phenotypic expression. Membranes were then transferred to LG agar containing tetracycline (25 µg/ml) and streptomycin (200 µg/ml) to select recipients which had aquired pBC16. Colonies were scored after 1 to 2 days and transcipients were purified by streaking for single colony isolation on appropriate selective media.

B. subtilis (natto) strains were tested for the ability to transfer pBC16 to B. anthracis. The pLS20$^+$ pBC16$^+$ strains, 3335 UM8, 3335 UM4 tr55K-1, and UM4 tr537B-15 were capable of transferring pBC16 to B. anthracis UM44-1 C9. Tc^r Str^r transcipients retained the auxotrophic marker (Ind$^-$) of the recipient strain. Transfer of pBC16 was confirmed by plasmid analysis of purified transcipients. All transcipients examined acquired pBC16. The pLS20$^-$ pBC16$^+$ strain, tr537B-16, did not transfer pBC16 to B. anthracis in two independent mating experiments.

Medium, mating and expression time, and the age of donor and recipient cells were varied to determine optimum mating conditions. Transcipients were obtained on PA, BHI, and LG media after 2 h on nonselective plates. The greatest number of transcipients was obtained on LG medium after 5 h of mating

and expression time. The age of donor and recipient transfer cultures before spreading on membranes was varied by testing 2-, 3-, 4-, and 5-h transfer cultures. Data presented in Table 8 show that the highest number of transcipients was obtained with 5-h cultures, although frequencies (No. of Tc^r transcipients per donor) did not vary significantly with the age of transfer cultures

Mechanism of plasmid transfer. To investigate the possibility that recipients were acquiring pBC16 by transformation we tested the sensitivity of plasmid transfer to DNase. B. subtilis (natto) 3335 UM8 and B. anthracis UM44-1 C9 transfer cultures were incubated with DNase (0.17 ml of a solution having 3 mg/ml in 0.01 M $MgSO_4$ was added to 5 ml of culture to give a final concentration of 100 µg/ml) for 15 min at 37C with shaking. Cultures were then mixed and spread onto membranes with an additional 10 µg of DNase. The number of transcipients obtained after incubation with DNase and $MgSO_4$ (4.4×10^3) did not differ significantly from the number of transcipients obtained after incubation with $MgSO_4$ alone (2.7×10^3).

To determine whether pBC16 transfer occurred by transduction, we tested the ability of a cell-free filtrate of B. subtilis (natto) 3335 UM8 to convert B. anthracis UM44-1 C9 to tetracycline resistance. The filtrate was prepared from a donor culture by filtering through an HA membrane. No Tc^r recipients were detected when recipient cells were spread with the filtrate on membranes.

Transfer of pBC16 from B. subtilis (natto) to B. anthracis in broth matings has not been observed. Tc^r transcipients were not detected when donor and recipient cultures were incubated at 37C with slow shaking during mating and expression time and then spread on selective medium.

Ability of B. subtilis (natto) to mate with various strains. Various Bacillus species were tested as recipients in matings with B. subtilis (natto). The results are shown in Table 9. The natto donor, 3335 UM8, was capable of transferring pBC16 to all species tested which included B. anthracis, B. cereus, B. thuringiensis, B. megaterium, and three strains of B. subtilis. Transfer of pBC16 to all of the above species has been documented by plasmid analysis of Tc^r transcipients.

IV. Determination of the size of pXO2 by restriction analysis

The size of pXO2 isolated from B. anthracis 6602 was estimated by digesting plasmid DNA with restriction endonucleases, EcoRI, PstI, and HaeIII, and determining the size of the individual fragments generated by comparing their migration in electrophoretic gels with that of DNA fragments of known size. DNA gel markers (BRL) which range from 0.5 kb to 12.2 kb in approximately 1 kb increments and lambda phage DNA which had been restricted with EcoR1 were used as standards.

The largest fragments generated by Pst1 and HaeIII were significantly larger than the largest standard fragment and this precluded an accurate estimation of their size. The sums of the fragment sizes from duplicate EcoR1 digestions were averaged to give an estimated size of 85.57 kb (approximately 57 Mdal). Table 10 lists the number and sizes of the individual EcoR1 fragments, as well as the HaeIII-generated fragments. Pst1 digestion generated 5 fragments (not listed in Table 10).

V. Transfer of pXO1 by the B. thuringiensis fertility plasmid pXO12

Curing B. anthracis of pXO12. In an earlier report (25) we showed that pXO12 was able to mobilize pXO1 among various B. anthracis strains. In the initial mating the proportion of Tc^r transcipients which simultaneously acquired pXO1 was quite low (2/475). In subsequent matings with one of these first $pXO1^+$ transcipients as a donor (UM44-1 C9 tr41G-1), the proportion of Tc^r transcipients which also picked up pXO1 increased significantly. To account for this increase in pXO1 transfer we hypothesized that pXO1 had been "primed" in the initial transfer event, thus allowing it to be transferred more frequently in subsequent matings. To facilitate plasmid DNA isolation in order to determine whether the "priming" involved any change in the physical structure of pXO1, it was necessary to obtain isolates of the low and high frequency transcipients which contained pXO1 alone. The B. anthracis transcipients Weybridge A UM2 tr244-1 (low frequency donor), UM44-1 C9 tr41G-1 (high frequency donor), A UM18 td2 C25-1 tr43G-11 (high frequency donor), and A UM23 C1-1 tr47G-34 (high frequency donor), all of which contained the three plasmids pXO1, pXO12, and pBC16, were incubated in the presence of novobiocin (1 µg/ml). After

several days of incubation the cultures were plated on L-agar and allowed to sporulate at 30°C. After sporulation the colonies were examined by phase contrast microscopy for the presence of crystals. A number of isolates were found which were Cry$^-$, and examination of their plasmid content confirmed that they were cured of pXO12. All of the pXO12$^-$ isolates examined were shown to harbor pXO1, but most of them retained pBC16. The pXO1$^+$ pXO12$^-$ transcipient clones which still contained pBC16 were cured of that plasmid by incubation overnight at 42°C. In this way we isolated B. anthracis A UM2 tr244-1 CN3H2, UM44-1 C9 tr41G-1 CN1H2, and A UM23 C1-1 tr47G-34 CN3 which contained pXO1 alone.

pXO1 plasmid DNA isolated from Weybridge A UM2 and the three transcipients listed above was digested with Pst1 or EcoR1 and the restriction fragment profile was examined by agarose gel electrophoresis. There were noticeable differences in the Pst1-generated band patterns of pXO1 from A UM2 and the low and high frequency pXO1 donors. pXO1 from A UM2 tr244-1 CN3H2 (low frequency) had lost the 5.4 kb Pst1 fragment found in pXO1 from UM2 and had acquired an 8.2 kb fragment, resulting in a net increase of 2.8 kb of DNA. Upon transfer of pXO1 from UM2 tr244-1 into UM44-1 C9 there were further alterations in the band pattern of pXO1. The 8.2 kb fragment of pXO1 in UM2 tr244-1 CN3H2 was missing in UM44-1 C9 tr41G-1 CN1H2 (high frequency) and UM23 C1-1 tr47G-34 CN3 (high frequency) and there were two new fragments, 7.5 kb and 4.5 kb in size.

Similar results were obtained with EcoR1 digestion. pXO1 from UM2 tr244-1 CN3H2 had lost the 7.95 kb fragment originally present in pXO1 from UM2 and had gained two new fragments of 6.45 kb and 4.15 kb, representing a net gain of 2.65 kb of DNA. Likewise there were further alterations in the high frequency donor A UM23 C1-1 tr47G-34 CN3. There was no apparent change in the 4.15 kb fragment, but the 6.45 kb fragment was replaced with a fragment of 10.6 kb. As revealed by the Pst1 and EcoR1 digests the alterations in pXO1 in the high frequency donors resulted in an additional 3.8 to 4.15 kb of DNA over that in pXO1 from A UM2 tr244-1 CN3H2, or a total of 6.6 to 6.9 kb additional DNA over that found in pXO1 isolated from UM2.

Source of acquired DNA in altered pXO1 plasmids. To determine whether the acquired DNA in the altered pXO1 plasmids was from pXO12 or a duplication of pXO1 DNA, pXO1 DNA from UM2, UM2 tr244-1 CN3H2, UM44-1 C9 tr41G-1 CN1H2, and A UM23 C1-1 tr47G-34 CN3 was probed with pXO12. pXO1 DNA was digested with Pst1, electrophoresed on agarose gels, and then transferred to Genescreen Plus

membranes. Plasmid pXO12 DNA was isolated from B. cereus 569R UM20-1 tr374B5 C1 and purified by CsCl gradient centrifugation. The DNA was then labelled with ^{32}P dCTP using the BRL nick translation kit as described by the supplier. The labelled pXO12 DNA was then used to probe the different pXO1 plasmids.

The unique fragments of the altered pXO1 plasmids hybridized with labelled pXO12, indicating that the acquired DNA in the altered plasmids originated from pXO12. There was no evidence of hybridization with any fragments of pXO1 from A UM2. In addition to the altered fragments, pXO12 also hybridized to the 6.7 kb Pst1 fragment in A UM2 tr244-1 CN3H2, UM44-1 C9 tr41G-1 CN1H2, and A UM23 C1-1 tr47G-34 CN3. This fragment did not appear to be altered from the analogous fragment of pXO1 from A UM2, but it is possible that the alteration is not large enough to effect a change in its migration. The fact that the intensity of the fragment on the autoradiogram was not as great as that of the obviously altered fragments would support the idea that there may be only a small piece of pXO12 on this fragment.

Test of the ability of strains containing altered pXO1 alone to mobilize pBC16. To determine whether the pXO12 DNA acquired by pXO1 encodes fertility functions, the various isolates which harbored altered pXO1 and pBC16 were mated with Tet^s recipients. None of the strains was able to mobilize pBC16. Thus, if the acquired DNA does carry transfer functions it does not contain all genes required for fertility. The role, if any, of the acquired DNA in the increased transfer frequency has yet to be determined. Whether the acquired DNA of the altered pXO1 plasmids is complementing transfer functions, or whether it enhances transfer by just supplying a region of homology with pXO12 are areas we hope to explore.

VI. Investigation of B. thuringiensis fertility plasmids, pXO13, pXO14, pXO15, and pXO16

We reported previously (26) on the identification of four new fertility plasmids in four different strains of B. thuringiensis. The transfer of plasmids by mating from four different B. thuringiensis subspecies to B. anthracis and B. cereus recipients was monitored by selecting for recipients which acquired the plasmid pBC16 (Tc^r). In addition to a random array of small plasmids, recipients also inherited a specific large plasmid from each B.

thuringiensis donor at a high frequency. These large plasmids (ca. 50-120 Mdal), pXO13, pXO14, pXO15, and pXO16, originating from B. thuringiensis subspecies morrisoni, toumanoffi, alesti, and israelensis, respectively, were demonstrated to be responsible for plasmid mobilization. Transcipients containing any of the above plasmids had donor capability, while B. thuringiensis donors cured of each of them were not fertile, indicating that the plasmids are necessary to confer conjugation functions. Confirmation that pXO13, pXO14, and pXO16 are self-transmissible was obtained by the isolation of fertile B. anthracis and B. cereus transcipients that contain only pBC16 and one of these plasmids. We have not been able to isolate a strain carrying pXO15 as the sole B. thuringiensis plasmid and therefore, have not obtained conclusive evidence that it is, in fact, self-transmissible. Results of our more recent work on these four fertility plasmids are reported here.

One of the most interesting questions arising from the discovery of six different self-transmissible plasmids in our laboratory regards the extent of relatedness among these plasmids. Their differences in mobility when electrophoresed in agarose gels indicated that they were not the identical plasmid simply isolated from different subspecies of B. thuringiensis, and examination of the DNA restriction patterns generated by the different plasmids has led to the conclusion that they are not related solely on the basis of duplications or deletions of DNA at one site. When some of the plasmids were probed for DNA homology by the Southern hybridization technique, considerable homology was found, but the existence of nonhomologous DNA restriction fragments for each plasmid confirmed their nonidentity. Their homology could have a basis in the presence of common gene sequences that encode their conjugation and transmission functions. However, the plasmids need to be probed with specific DNA fragments associated with fertility functions before any conclusions may be made about the origins of their DNA homology. Research in this direction is now in progress in our laboratory. We have isolated various transfer-deficient mutants of pXO12. By comparing the restriction patterns of these plasmids to wild type pXO12, we are now in a good position to identify particular fragments involved in plasmid transmission.

Correlation of parasporal crystal production with specific plasmids. In many B. thuringiensis subspecies, the formation of parasporal crystals is associated wtih specific plasmids. Our work with the fertility plasmid, pXO12, from subsp. thuringiensis has shown it carries genes for paraspsoral crystal

synthesis. However, none of the four new B. thuringiensis fertility plasmids identified in this study, i.e., pXO13, pXO14, pXO15, or pXO16 carries crystal toxin genes since B. cereus and B. anthracis transcipients containing any of these plasmids do not produce crystals during sporulation. Furthermore, cured derivatives of the B. thuringiensis strains originally carrying one of the fertility plasmids were still Cry^+, which indicated that pXO13, pXO14, pXO15, or pXO16 was not necessary for crystal synthesis either. An interesting finding resulted from examining cured B. thuringiensis BIS UM1 subsp. israelensis isolates for their production of parasporal crystals. We noticed that derivatives which were cured of the third largest plasmid, named pXO39 (ca. 90 Mdal), differed from the parent strain in the parasporal inclusions produced. The former strains showed the presence of a tiny inclusion in the cytoplasm of sporulating cells, while pXO39-containing strains produced a large crystalline inclusion that was polymorphic in shape, in addition to a tiny inclusion. Often pXO39-containing cells produced several large as well as tiny inclusions. Variants which were cured of pXO39 occurred spontaneously fairly frequently. When B. thuringiensis BIS UM1 was streaked on solid media, such variants were easily identified owing to their altered colony morphology.

Mobilization of Bacillus anthracis plasmids by pXO14. As previously reported, the B. thuringiensis subsp. thuringiensis fertility plasmid pXO12 can mobilize the B. anthracis toxin and capsule plasmids, pXO1 and pXO2 (2, 24, 25, 26). We have since demonstrated that the B. thuringiensis 4059 subsp. toumanoffi plasmid pXO14 will mobilize pXO1 and pXO2 from B. anthracis to plasmid-cured B. anthracis or B. cereus recipients more efficiently than pXO12. To detect the transfer of pXO1 to recipients, transcipients were picked to CA-agarose media containing antiserum to the anthrax toxin. The plasmids were then extracted from colonies which produced a halo in this medium to confirm their acquisition of a plasmid with the mobility of pXO1. When the pXO14-containing B. anthracis donor, UM44-1 tr169A-4 was used, 6% of the Tc^r transcipients resulting from a mating with a plasmid-cured B. anthracis recipient acquired pXO1. The transcipient Weybridge A UM23C1-1 tr357A-54 contained a plasmid band which migrated slower than pXO1, and was not characteristic of either of its parent strains. It was noticed that many of the transcipients derived from matings performed with a pXO1-containing B. anthracis strain as the donor contained plasmids which did not correspond to either pXO1 or the donor plasmids. These plasmid variants could code for protective antigen

production in many instances. There are many reports in the literature presenting evidence of repetitive sequences and transposon-like elements present on B. thuringiensis plasmids (11, 13-16). Therefore, the variant plasmids might result from transposition of sequences from B. thuringiensis plasmids to pXO1. They might also simply represent amplification of DNA sequences within pXO1 itself. A more exciting possibility, from the point of view of the mechanism of the conjugation system, is that these plasmids result from imprecise resolution of an intermediar cointegrate formed between the self-transmissible plasmid and pXO1 during transfer. We are investigating this by probing such variant plasmids for the presence of B. thuringiensis plasmid sequences.

By mating a pXO14-containing donor with B. anthracis 4229 a transcipient which contained pXO14, pXO2, and pBC16 was constructed. When this strain was used as a donor to a B. cereus 569 UM20-1 recipient, 1% of the transcipients displayed the Cap^+ phenotype characteristic of cells containing pXO2. Cap^+ transcipients were selected on the basis of resistance to the bacteriophage CP-54. Plasmid analysis confirmed that such transcipients had acquired pXO2. The self-transmissible plasmid pXO16 was also able to mobilize pXO2 to recipients, though the frequency was lower. With pXO16-containing donors, only 0.25% of the To^r transcipients inherited pXO2.

DNA Homology among Bacillus thuringiensis fertility plasmids. Currently our laboratory has identified six different B. thuringiensis self-transmissible plasmids. Since these plasmids are related in terms of their conjugative functions, the question arises whether they contain common sequences encoding these functions. As a preliminary step toward gaining some insight into this possibility, we decided to probe some of the plasmids for the presence of homologous DNA.

To look for homology among the plasmids pXO11, pXO12, pXO13 and pXO14, the plasmid DNAs were digested with the restriction endonuclease PstI and transferred after electrophoresis to a nylon membrane. We used Southern hybridization (20) to probe the immobilized DNA with ^{32}P-labelled pXO11 DNA. The four fertility plasmids showed considerable homology, but each displayed certain unique restriction fragments. The restriction patterns also indicated that these plasmids were not simple derivatives of each other.

Conjugations between strains containing different fertility plasmids. Incompatibility among various plasmids can be a measure of their relatedness. Therefore, we wished to determine whether the fertility plasmids could be

separated into incompatibility groups. Also, we wanted to determine whether there were any entry exclusion effects in matings between two strains containing the same fertility plasmid. To do this, strains containing the same or different self-transmissible plasmids were mated, the numbers of Tc^r transcipients per ml were determined, and several transcipients from relevant matings were screened for their plasmid content.

It was decided that for the sake of consistency when comparing the different plasmid-containing donors, it would be desirable to adhere to intraspecies matings, using comparable B. anthracis donors and recipients whenever possible. This was not possible in the case of matings involving pXO15-containing donors or recipients, since we were unable to construct appropriate B. anthracis strains. Hence in experiments involving pXO15, matings were done using the B. thuringiensis YAL UM1 donor, or between B. cereus donors and a pXO15-containing B. cereus Tet^s recipient. The numbers of Tc^r transcipients obtained in these matings are shown in Table 11. Based on the frequencies of pBC16 transfer observed, it is clear that pXO12, pXO13, pXO14, pXO15, and pXO16 do demonstrate entry exclusion effects. There was a diminished frequency of pBC16 transfer between donors and recipients which contained the same fertility plasmid. This inhibition resulted in about 20 to 30-fold lower frequencies when donor and recipient strains both contained pXO12, pXO13, or pXO16, while a pXO14-containing donor transferred pBC16 at a 60-fold lower frequency to a recipient containing pXO14. With the pXO15-containing strains there was a greater inhibition of transfer of pBC16 to recipients containing pXO15, i.e., the frequency was reduced about a 1000-fold.

Several transcipients from the matings between the strains shown in Table 11 were purified and their plasmids were extracted. The results of this plasmid analysis are summarized in Table 12. It was found that pXO13 and pXO14 could each coexist in the same strain with pXO15 or pXO16, but the data suggest that pXO14 and pXO13 are not compatible. The maintenance of pXO14 seemed to be dominant to pXO13 maintenance. pXO16 was also found to be compatible with pXO15. pXO11 and pXO12 could each be stably maintained along with pXO16. The compatibility of pXO11 with pXO14, and of pXO12 with pXO13 could not be determined. Their mobilities on an agarose gel are very similar and so their individual presence in transcipients was not resolvable. All the 16 transcipients examined from a mating between a pXO12 containing donor and a recipient with pXO13 were Cry^+, which indicated that they do contain pXO12.

VII. Physical and genetic characteristics of B. thuringiensis fertility plasmids pXO11 and pXO12

We have continued research toward characterization of the Bacillus mating system encoded by B. thuringiensis fertility plasmids. We have chosen to concentrate on the fertility plasmids pXO11 and pXO12, both of which were found in strain 4042A of B. thuringiensis subsp. thuringiensis. In addition to conjugal transfer functions, pXO12 also codes for the synthesis of parasporal crystals (Cry^+). Both fertility plasmids are capable of promoting their own transfer as well as the transfer of a wide range of nonconjugative plasmids.

Considerable effort has been devoted toward understanding the molecular relationship between the two B. thuringiensis plasmids, pXO11 and pXO12. Results from Southern hybridization experiments have shown that there is considerable homology between pXO11 and pXO12. Experiments designed to investigate entry exclusion and incompatibility properties of these two plasmids suggest that pXO11 and pXO12 code for different entry exclusion and replication systems. Therefore, it appears as if pXO11 and pXO12 are distinct plasmids yet they do share some common gene sequences.

Restriction analysis of three independent Cry^+ Tra^- deletion derivatives of pXO12 has indicated that gene(s) responsible for conjugal transfer ability are located on a 70 kilobase PstI fragment of pXO12. Restriction analysis of several Cry^- Tra^+ deletion mutants of pXO12 suggest that pXO11 may be a naturally occurring deletion derivative of pXO12. Further evidence in support of this proposed relationship is the detection of plasmids similar in size and phenotype (Cry^- Tra^+) to wild-type pXO11 in transcipients derived from a partially cured derivative of strain 4042A which only harbors the fertility plasmid pXO12.

Molecular homology of pXO11 and pXO12. In view of the ubiquity of large molecular weight self-transmissible plasmids among naturally occurring strains of B. thuringiensis (3), it was of interest to determine the extent of homology between pXO11 and pXO12. Restriction endonuclease analysis and Southern hybridization experiments were employed for this purpose. The results of these experiments are discussed below.

For Southern hybridization experiments, purified pXO11 DNA was labelled by

nick translation with ^{32}P and then hybridized to nitrocellulose blots of PstI and EcoRI restriction digests of pXO11 and pXO12. Autoradiography revealed that five of the seven pXO12 fragments produced by PstI digestion hybridized to radiolabelled pXO11 DNA. Similarly, ten of the twenty pXO12 fragments generated from EcoRI digestion hybridized to pXO11 DNA. Although these results suggest that pXO11 shares considerable homology with pXO12, the large extent of homology may be attributable to DNA sequences unrelated to conjugal transfer functions. Evidence to support this idea is discussed below.

Restriction endonuclease analysis of pXO11 and pXO12. The number and pattern of DNA fragments generated from 11 different restriction endonucleases has been examined for both pXO11 and pXO12. The results of this survey are summarized in Table 13. pXO11 and pXO12 both contained unique cleavage sites for BamHI, BglI and SmaI. EcoRI, HindIII and HaeIII generated complex patterns of bands, some of which were too faint on the stained gels to permit precise enumeration. Restriction endonucleases resulting in less complex patterns included: PstI, KpnI, ClaI, XbaI and SalI. Examination of the restriction patterns generated by all enzymes revealed that the restriction patterns of pXO11 and pXO12 were quite different, suggesting that pXO11 is not a simple deletion derivative of pXO12.

Of the five hexanucleotide-specific restriction enzymes examined i.e., PstI, KpnI, ClaI, XbaI and SalI, all but one, XbaI, generated one large molecular weight fragment (>30 kb) in addition to smaller fragments. For example, PstI yielded a 47.8 and a 70.0 kb fragment from pXO11 and pXO12, respectively. pXO12 yielded a 59.8 kb fragment with ClaI and a 76.0 kb fragment with SalI. These data suggest that there may be a region on pXO11 and pXO12 which contains relatively few cleavage sites for restriction endonucleases with hexanucleotide specificity.

Sizes of pXO11 and pXO12. The sizes of pXO11 and pXO12 were determined by summation of fragments generated by various restriction endonucleases (Table 14). For each plasmid at least two restriction enzymes were used, and the results presented represent averages of two determinations. According to the ClaI digestions, the sizes of pXO11 and pXO12 were found to be 84 kb (56 megadaltons) and 112.5 kb (75 megadaltons), respectively. Although the estimated sizes resulting from the three determinations were in good agreement, estimations from the ClaI digestions were probably the most accurate since all the fragments fell within the size-range of the standard curve. By averaging

the results from the various determinations the values of 84.5 kb for pXO11 and 110.4 kb for pXO12 were obtained.

Deletion derivatives of pXO11 and pXO12. The novelty of a Bacillus conjugal transfer system leads to many interesting questions. One of the most interesting is how much DNA is devoted to this process and how is the information arranged on the fertility plasmids. One approach to mapping the transfer genes of pXO11 and pXO12 is by restriction analysis of transfer-defective mutants of these plasmids. Comparative analysis of transfer deficient (Tra^-) and wild-type Tra^+ plasmids should permit the preliminary assignment of conjugal transfer function(s) to specific restriction fragment(s).

To study the genetic and physical organization of pXO11 and pXO12 via restriction analysis, we have taken advantage of a growing collection of deletion mutants derived from wild-type pXO11 and pXO12. The deletion mutants were isolated from strains that had been grown at 42°C or from transcipients derived from matings in which the donors were B. anthracis strains harboring wild-type pXO11 or pXO12. For mutants to be most useful for genetic mapping, they should be totally defective in conjugal transfer. Although the majority of our pXO12 deletion mutants are only impaired in transfer ability, three that are Cry^+ Tra^- have been obtained. To date, no pXO11 derivatives have been obtained which are completely Tra^-.

The relevant characteristics, origins and restriction patterns of some of these deletion mutants are summarized below. The following three types of deletion plasmids will be discussed: (1) partially transfer deficient pXO12 mutants, i.e., plasmids that transfer pBC16 at lower frequencies than wild-type pXO12; (2) completely transfer-negative pXO12 deletion mutants; and (3) a pXO11 deletion derivative with enhanced transfer ability.

Partially transfer defective deletion mutants of pXO12. We have tentatively grouped seven pXO12 Tra^+ deletion mutants into two classes according to their size as reflected in their mobility in 0.6% agarose gels (Table 15). Class I consisted of three Cry^+ Tra^+ deletion mutants (pXO12Δ223, pXO12Δ379, and pXO12Δ388) which appeared to be slightly smaller than pXO12. Class II contained four Cry^- Tra^+ mutants (pXO12Δ44, pXO12Δ237, pXO12Δ373, and pXO12Δ410) which are approximately the same size as wild-type pXO11.

Although the number of Tra^+ deletion mutants of pXO12 thus far examined is small, the available information suggests some interesting generalizations concerning deletion formation in pXO12 and on the molecular relationship between

pXO11 and pXO12: (1) The occurrence of spontaneous deletions did not appear to be a random process since the mutants fit into two distinct size classes; (2) Although the crystal phenotype was conserved within the two classes of deletion mutants, the transfer efficiency varied within each deletion class, i.e., some mutants of the same size were more efficient donors of pBC16 than others; (3) More than half of the Tra^+ deletion plasmids isolated were approximately the size of wild-type pXO11 and were similar to pXO11 in transfer efficiency. The latter observation may provide evidence to support the idea that pXO11 is actually a deletion derivative of pXO12. Further evidence for this hypothesis is presented below.

Restriction analysis was carried out on two Class I Tra^+ deletion mutants (pXO12Δ379 and pXO12Δ388) and two Class II Tra^+ deletion mutants (pXO12Δ237 and pXO12Δ410) using the following restriction enzymes: PstI, XbaI, ClaI, KpnI and SalI. Examination of the restriction patterns of wild-type pXO12 and the pXO12 deletion derivatives allowed the identification of particular fragment(s) which had either suffered a deletion or had been lost completely in the mutant plasmids. The four mutants exhibited two types of restriction patterns; Class I mutants gave pXO12-like patterns and Class II mutants generated pXO11-like patterns. The Class I mutants have small deletions in the largest pXO12 fragment.

Transfer defective deletion mutants of pXO12. The origins and relevant characteristics of three Cry^+ Tra^- deletion derivatives of pXO12, pXO12Δ43, pXO12Δ47, and pXO12Δ203, are summarized in Table 16. Two of them, pXO12Δ43 and pXO12Δ47, appeared to be slightly smaller than wild-type pXO12, while the third mutant, pXO12Δ203, was approximately the same size as wild-type pXO11.

Restriction analysis of the three Tra^- deletion derivatives described above was carried out using the restriction endonuclease PstI. Preliminary results indicate that all three independent Cry^+ Tra^- mutants have a deletion in the 70.0 kb PstI fragment. These results suggest that the 70 kb PstI fragment contains gene(s) required for conjugal transfer ability. The results (described above) of restriction analysis of two of the partially transfer defective pXO12 mutants, pXO12Δ379 and pXO12Δ388, lend further support for this hypothesis; both of these mutants also have deletions in the 70.0 kilobase PstI fragment.

Characterization of a pXO11 deletion derivative. To date, only one deletion derivative of pXO11 has been isolated (pXO11Δ418 in Table 16). It is very interesting that the deleted plasmid is a more effective fertility plasmid

than its parent, wild-type pXO11; pXO11Δ418 transferred pBC16 at a 10^2- to 10^3-fold higher frequency than did wild-type pXO11. One possible explanation for this phenomenon is that the deleted DNA sequences code for a repressor or a repressor binding site involved in regulation of pXO11 transfer function(s). Thus, pXO11 may normally exist in a repressed state and loss of gene(s) involved in regulation generates plasmids such as pXO11Δ418 which are de-repressed with respect to fertility.

PstI digestion of pXO11Δ418 produced a pXO11-like restriction pattern except that three fragments were lost (11.5, 3.2 and 2.2 kb) and one new fragment was gained (approximately 4 to 5 kb). Thus, pXO11Δ418 resulted from a net loss of approximately 13 kilobases.

Discussion of above results with pXO11 and pXO12. Results from Southern hybridization experiments indicate that the two fertility plasmids, pXO11 and pXO12, share considerable homology. Analogous results have been reported for gram-negative fertility plasmids. Conjugative plasmids of gram-negative bacteria that specify similar conjugation systems have been found to exhibit 40 to 80% homology while plasmids coding for different systems show less than 10% homology (28). This high degree of homology is attributable to the large amount of DNA devoted to conjugation. In light of these findings, it seems possible that the homology shared by pXO11 and pXO12 may be attributable to their transfer gene(s). However, it also seems possible that much of the homology observed may not be related to DNA sequences involved in conjugal transfer functions. The molecular relationship of B. thuringiensis plasmids in general has been investigated by others (12, 13). Those studies revealed that there is both interstrain and interspecies DNA homology among large B. thuringiensis plasmids. The homology has been attributed to the presence of multiple copies of (1) parasporal crystal genes, (2) inverted repeat sequences, and (3) small cryptic elements with transposon-like properties (11, 14, 15, 16). Thus, in light of the molecular relatedness among B. thuringiensis plasmids in general, much of the homology observed between pXO11 and pXO12 might be ascribable to sequences other than those involved in conjugation.

To more accurately assess the homology of the transfer genes carried by pXO11 and pXO12, hybridization experiments will be repeated using a restriction fragment of pXO12 that carries sequences necessary for conjugal transfer ability. Such a fragment, which will be identified by comparative restriction analysis of transfer defective mutants and wild-type pXO12, would provide a very

specific probe for examining the transfer regions of pXO11 and pXO12 as well as other B. thuringiensis fertility plasmids currently under investigation in our laboratory.

Although Southern hybridization experiments suggest that pXO11 and pXO12 are closely related, results of restriction analyses appear to contradict this finding. One possible explanation for this discrepancy is that the two plasmids consist largely of homologous sequences arranged in a different order on each plasmid. The existence of inverted repeats on various B. thuringiensis plasmids as described by Kronstad and Whiteley (11) and by Lereclus et al. (15) may afford the means of accomplishing DNA rearrangements in these plasmids. For example, it is known that reciprocal recombination between a pair of inverted repeats results in the inversion of the region between them.

Results of restriction analyses suggest that there may be regions on pXO11 and pXO12 which contain relatively few cleavage sites for restriction endonucleases with hexanucleotide specificity. The same observation has been reported for the gram-negative self-transmissible plasmid F and the related F-like R factors (1, 18, 29,). Similarly, Meyers et al. (17) have reported that there are relatively few restriction sites in the broad host range plasmid, RK2, and have proposed that there is strong selective pressure for loss of endonuclease cleavage sites in broad host range plasmids which are often exposed to a variety of restriction endonucleases. An analogous situation may exist for the B. thuringiensis fertility plasmids, pXO11 and pXO12. Although they may not be considered broad host range plasmids, their maintenance and expression in several Bacillus species may necessitate a paucity of cleavage sites in the transfer and replication regions of these plasmids.

VIII. Restriction analysis of virulence plasmids from various Bacillus anthracis strains.

The plasmid pXO1 from various B. anthracis strains was examined by restriction endonuclease analysis to determine the banding patterns. EcoRI restriction digests of pXO1 from PM-36 R1, M UM6, Ames ANR-1 and New Hampshire NNR-1 yielded the same banding pattern. This pattern appeared to be contained within the restriction pattern of pXO1 from the other strains tested. The EcoRI digest of pXO1 from strain UM44-1 contained four additional or altered bands

when compared to the basal pattern produced by pXO1 from the four strains above. These fragments were 6.6, 6.3, 3.02 and 1.16 kb. Although the 1.16 kb fragment was present in digests of pXO1 from the four strains mentioned above, it was highly intensified in brightness in pXO1 from M44-1 indicating multiple copies of the fragment. The EcoRI digests of pXO1 from the Weybridge wild-type strain and the two Weybridge mutant strains, A M18 and A M23, each contained only one additional band not found in the basal pattern produced by pXO1 from Vollum PM-36 R1, M UM6, ANR-1, and NNR-1. This fragment was 6.6 kb in size.

The PstI restriction digests of pXO1 from PM-36 R1, New Hampshire NNR-1, and M UM6 yielded the same banding pattern; digests of pXO1 from ANR-1 appeared to have a very minor alteration in one fragment. pXO1 from Weybridge M44-1 produced at least three additional bands not found in PstI digests of pXO1 from PM-36 R1, NNR-1, and MR3.

pXO2 preparations from various B. anthracis strains were also examined by EcoRI restriction endonuclease analysis to compare the banding patterns. pXO2 from the Pasteur strains 4229 and 6602 and strain AmesΔ1 produced identical patterns of fragments. The restriction patterns of pXO2 from M UM2 and NHΔ1 were identical and showed one altered fragment compared to pXO2 from the other strains. The size of the unique fragment was estimated to be 4.95 kb and the size of the fragment which it apparently replaced was estimated to be 4.89 kb. This difference may result from the addition of 50 to 60 base pairs to a fragment which is common to pXO2 from the other strains.

These results suggest that pXO1 and pXO2 are generally well conserved among the various strains examined, with only small differences revealed by EcoRI and PstI restriction analyses.

TABLE 1. List of bacterial strains

Strain	Characteristics and/or source
B. anthracis	
4229 (Pasteur)	Cap^{+c} Tox^- (pXO2) [B. Ivins]
4229 UM12	Nal^r by UV of 4229, (pXO2)
4229 UM12 tr407A-7	Cap^+ Tc^r Nal^r (pXO2, pXO14, pBC16)
6602 (Pasteur)	Cap^{+c} Tox^- (pXO2) [B. Ivins]
6602 tr172B-2	Cap^{+c} Cry^+ Tc^r (pXO2, pXO12, pBC16)
6602 R1	Cap^- $pXO2^-$, spontaneous from 6602
6602 R4	Cap^- (pXO2), spontaneous from 6602
6602 R4 C1	Cap^- $pXO2^-$
6602 R4 C1 tr16K-1	Cap^{+c} Tc^r (pXO12::pXO2, pBC16)
6602 R5	Cap^- (pXO2), spontaneous from 6602
6602 R5 C1	Cap^- $pXO2^-$
6602 R5 C1 tr17K-1	Cap^{+c} Tc^r (pXO12::pXO2, pBC16)
PM-36	Vollum strain, (pXO1, pXO2) [MRE]
PM-36 R1	Cap^- $pXO2^-$ spontaneous from PM-36, (pXO1)
AmesΔ1	Cap^{+c} Tox^- $pXO1^-$ (pXO2) [B. Ivins]
Ames ANR-1	Cap^- Tox^+ $pXO2^-$ (pXO1) [B. Ivins]
New Hampshire NHΔ1	Cap^{+c} Tox^- $pXO1^-$ (pXO2) [B. Ivins]
New Hampshire NNR-1	Cap^- Tox^+ $pXO2^-$ (pXO1) [B. Ivins]
M	Cap^{+a} Tox^+ (pXO1, pXO2.1)
M UM1	M *str-1* (pXO1, pXO2.1)
M UM2	UM1 cured of pXO1 by growth at 42^o, Str^r (pXO2.1)
M UM2 tr24K-5	Cap^{+a} Str^r Tc^r (pXO2.1, pXO14, pBC16) See text

TABLE 1. (continued)

M UM3	Spontaneous Cap^{+c} mutant of UM2 (pXO2.1)
M UM4	Spontaneous Tox^- Cap^- from M, no plasmids
M UM15	UM4 td to Cap^{+c} from 6602, (pXO2)
M UM22	UM4 td to Cap^{+c} from 6602, (pXO2)
M UM5	$pXO2.1^-$, Spontaneous Cap^- from M
M UM6	$pXO2.1^-$, Spontaneous Cap^- from M
Weybridge	Cap^- Tox^+ (pXO1) $pXO2^-$ [MRE]
Weybridge A	Colonial variant of Weybridge, (pXO1)
A UM2	Ind^- by UV of Weybridge A, (pXO1)
A UM2 tr244-1	Ind^- To^r Cry^+ (pXO1, pXO12, pBC16)
A UM2 tr244-1 CN3	$pXO12^-$ of tr244-1 with novobiocin, (pXO1, pBC16)
A UM2 tr244-1 CN3H2	$pBC16^-$ of tr244-1 CN3 at 42^o, (pXO1)
A UM17	Ade^- by UV of Weybridge A, (pXO1)
A UM17 tr57B-6	Ade^- To^r Cry^+ (pXO1, pXO12, pBC16)
A UM18	pyrA by UV of Weybridge A, (pXO1)
A UM18 td2	UM18(pBC16) by transduction
A UM18 td2 C25	UM18 td2 $pXO1^-$ $pBC16^-$ by growth at 42^o
A UM18 td2 C25-1	Spontaneous Str^r of td2-C25
A UM18 td2 C25-1 tr43G-11	To^r Cry^+ Str^r (pXO1, pXO12, pBC16)
A UM18 td2 C25-1 tr43G-11 CN1	Cured of pXO12 with novobiocin, (pXO1, pBC16)
A UM18 td2 C25-1 tr43G-11 CH2	$pXO1^-$ of tr43G-11 by growth at 42^o, Cry^+ Tra^- (pXO12Δ43, pBC16)
A M18 td2 C25-1 tr43G-11 C5	$pXO1^-$ of tr43G-11 CH2 by growth at 42^o, $pBC16^-$ Cry^+ (pXO12Δ43
A UM23	Ura^- by UV of Weybridge A (pXO1)
A UM23 C1	Ura^-, $pXO1^-$ of A UM23
A UM23 C1 tr66G-1	Ura^- To^r Cry^+ Cap^{+c} (pXO12::pXO2, pBC16)

TABLE 1. (continued)

A UM23 C1-1	str-2 by UV of A UM23 C1
A UM23 C1-1 tr47G-34	Ura^- To^r Cry^+ Tox^+ (pXO1, pXO12, pBC16)
A UM23 C1-1 tr47G-34-CH3	Ura^- Str^r Cry^+ Tra^- (pXO12Δ47,pBC16)
A UM23 C1-1 tr47G-34-C1	Tet^s, $pBC16^-$ of tr47G-34 CH3 by growth at 42°, Cry^+ (pXO12Δ47)
A UM23 C1-1 tr47G-34 CM1	Cry^- $pXO12^-$ of tr47G-34 with novobiocin, (pBC16)
A UM23 C1-1 tr47G-34 CM3	Tet^s, $pBC16^-$ of tr47G-34 with novobiocin, (pXO1)
A UM23 C1-1 tr336A-4	Ura^- To^r (pXO16, pBC16)
A UM23 C1-1 tr338A-1	Ura^- To^r (pXO14, pBC16)
A UM23 C1-1 tr357A-16	Ura^- Str^r (pXO1, pXO14, pBC16)
A UM23 C1-1 tr357A-54	Ura^- Str^r (pXO1, pXO14, pBC16)
A UM23 C1-1 tr359A-1	Ura^- To^r (pXO13, pBC16)
A UM23 C1-2	A UM23 C1 rfm-1 by td from A M693 His^- rfm-1
A UM23 C1-2 tr30-K4	Cap^{+a} Rif^r To^r (pBC16, see text)
A UM23 C1-2 tr30K-5	Cap^{+a} Rif^r To^r (pXO2.1Δ1, pXO14, pBC16)
A UM23 C1-2 tr30K-6	Cap^{a+} Rif^r To^r (pBC16, see text).
A UM23 C1-2 tr43K-3	Cap^{+a} Ura^- Rif^r To^r (pXO2.1Δ1, pBC16)
A UM23 C1-2 tr43K-3 C1	$pBC16^-$ of tr43K-3 Cap^{+a} Ura^- Rif^r (pXO2.1Δ1)
A UM23 C1-2 tr43K-5	Cap^- Ura^- Rif^r To^r (pBC16)
A UM23 C1-2 tr418B-1	Ura^- To^r Tra^+ Rif^r (pXO11Δ418, pBC16)
A UM23 C1-2 tr418B-1 C1	Tet^s $pBC16^-$ of tr418B-1 by growth at 42°, Rif^r (pXO11Δ418)
A UM23 C2	$pXO1^-$ of A UM23, Ura^-
A UM23 C2 tr44B-9	Ura^- To^r Cry^- Tra^+ (pXO12Δ44, pBC16)
A UM23 C2 tr45B-12	Ura^- To^r (pXO11, pBC16)
A UM23 C2 tr60B-1	Ura^- To^r Cry^+ (pXO12, pBC16)

TABLE 1. (continued)

A UM23 C2 tr96B-3	Ura^- Tc^r (pXO11, pBC16)
A UM23 C2 tr96B-3-C19	$pBC16^-$ of tr96B-3, Ura^- Tet^s (pXO11)
A UM23 C2 tr237-10	Ura^- Tc^r Cry^+ (pXO12, pBC16)
A UM23 C2 tr237-10-C29	Tet^s $pBC16^-$ of tr237-10 by growth at 42^o, Ura^- Cry^+ (pXO12)
A UM23 C2 tr237-10 C19-5	Ura^- Tet^s Cry^+ (pXO12Δ237)
A UM23 C2 tr237-10 `` 5 td1	Tc^r by td of tr237-10 C19-5, Ura^- Cry^+ Tra^+ (pXO12Δ237, pBC16)
A UM23 C2 tr379B-2	Ura^- Tc^r Cry^+ Tra^+ (pXO12Δ379, pBC16)
A UM23 C2 tr379B-2 C1	Tet^s $pBC16^-$ of tr379B-2 by growth at 42^o, Cry^+ (pXO12Δ379)
A UM23 C2 tr388B-5	Ura^- Tc^r Cry^+ Tra^+ (pXO12Δ388, pBC16)
A UM23 C2 tr388B-5 C1	Tet^s $pBC16^-$ of tr388-5 by growth at 42^o, Cry^+ (pXO12Δ388)
A UM23 C2 tr410B-1	Ura^- Tc^r Cry^- Tra^+ (pXO12Δ410, pBC16)
A UM23 C2 tr410B-1 C2	Tet^s $pBC16^-$ of tr410B-1 at 42^o, (pXO12Δ410)
UM44	Ind^- by UV of Weybridge, (pXO1)
UM44-1	Str^r by UV of UM44, (pXO1)
UM44-1 tr169A-4	Ind^- Str^r Tc^r (pXO1, pXO14, pBC16)
UM44-1 tr203-1	Ind^- Str^r Tc^r Cry^+ (pXO1, pXO12, pBC16)
UM44-1 C9	$pXO1^-$ at 42^o, Ind^- Str^r
UM44-1 C9 tr41G-1	Ind^- Tc^r Str^r Cry^+ Tox^+ (pXO1, pXO12, pBC16)
UM44-1 C9 tr41G-1 CN1	$pXO12^-$ of tr41G-1 with novobiocin, (pXO1, pBC16)
UM44-1-C9 tr41G-1 CN1H2	$pBC16^-$ of tr41G-1-CN1 at 42^o, (pXO1)
UM44-1 tr203-7	Ind^- Str^r Tc^r Cry^+ Tra^+ (pXO1, pXO12, pBC16)
UM44-1 tr203-23	Ind^- Str^r Tc^r Cry^+ (pXO1, pXO12, pBC16)
UM44-1 tr203-23 C1-2	Str^r Cry^+ (pXO1, pXC12Δ223)

TABLE 1. (continued)

UM44-1 tr203-23 C1-2 td1	Tc^r by td of tr203-23 C1-2, Cry^+ Tra^+ (pXO1, pXO12Δ223, pBC16)
UM44-1 tr203-23 C1-4	Ind^- Str^r Tet^s Cry^+ $pBC16^-$ (pXO1, pXO12Δ203)
UM44-1 tr203-23 C1-4 td1	Tc^r by td of tr203-23 C1-4, Cry^+ Tra^- (pXO1, pXO12Δ203, pBC16)
UM44-1 tr203-23 C1-413	Ind^- Str^r Tet^s Cry^+ $pBC16^-$ (pXO12Δ203)
UM44-1 tr373B-4	Ind^- Str^r Tc^r Cry^- Tra^+ (pXO1, pXO12Δ373, pBC16)
B. cereus	
569	wild type (pXO3, pXO4, pXO5) [NRRL]
569 UM20	Ant^- by UV of 569 (pXO3)
569 UM20-1	Str^r by UV of UM20 (pXO3)
569 UM20-1 td10	Ant^- Cap^{+c} by td from 6602 (pXO2, pXO3)
569 UM20-1 tr60G-10	Ant^- Str^r Tc^r Cap^+ Cry^+ (pBC16, pXO12::pXO2)
569 UM20-1 tr374B-5	Ant^- Str^r Tc^r Cry^+ Tra^+ (pXO12, pBC16)
569 M20-1 tr374B-5 C1	Tet^s $pBC16^-$ of tr374B-5 by growth at 42^o, Cry^+ (pXO12)
569 UM20-1 tr412A-1	Ant^- Str^r Tc^r (pXO2, pXO3, pXO14, pBC16)
569 UM34	Ura^- by UV of 569 (pXO3, pXO5)
569 UM34 tr163A-1	Ura^- Tc^r (pXO3, pXO5, pXO16, pBC16)
569 UM47	Ade^- by UV of 569 (pXO3, pXO4, pXO5)
569 UM47 tr292A-11	Ade^- Tc^r (pXO3, pXO4, pXO5, pXO13, pBC16)
569 UM47 tr320A-3	Ade^- Tc^r (pXO3, pXO4, pXO5, pXO14, pBC16)
B. thuringiensis	
4042A	subsp. thuringiensis, Cry^+ (pXO11, pXO12)
4042A UM8	Ade^- by UV of 4042A, Cry^+ (pXO11, pXO12)
4042A UM8 td2	Tc^r by td of UM8, (pXO11, pXO12, pBC16)
4042A UM8-13	Osp mutant of UM8 (pXO11, pXO12)

TABLE 1. (continued)

4042A UM8-13 td1	Tc^r by td of UM8-13 (pXO11, pXO12, pBC16)
4042A UM8-13 td1-A	Spontaneous $pXO12^-$ from UM8-13 td1 (pXO11, pBC16)
4042A UM2-1	pyrA Str^r (pXO11, pXO12)
4049	subsp. morrisoni (pXO13) [NRRL]
4049 UM1	Tc^r by td of 4049, (pXO13, pBC16)
4059	subsp. toumanoffi, Cry^+ [NRRL]
4059 UM1	Tc^r by td of 4059, (pXO14, pBC16)
BIS	subsp. israelensis (pXO16) [M. deBarjac]
BIS UM1	Tc^r by td of BIS, (pXO16, pBC16)
YAL	subsp. alesti (pXO15) [A. Yousten]
YAL UM1	Tc^r (pXO15, pBC16)
B. megaterium	
PV229	Leu^- Str^r Pep^- Meg^- [P. Vary]
B. subtilis	
168	trpC2 [M. Fox]
IA510	r^-m^- str-2 recE Arg^- Thr^- Leu^- [D. Dean]
PY143	Cm^r MLS^r (pTV1) [P. Youngman]
W23	Str^r [M. Fox]
B. subtilis (natto)	
3335	Pga^+ (pLS19, pLS20) [T. Hara]
3335 UM4	Spontaneous Pga^- $pLS19^-$ from 3335, (pLS20)
3335 UM5	Rif^r by UV of UM4 (pLS20)
3335 UM8	Tc^r by tf of UM4, (pLS20, pBC16)
3335 UM4 tr55K-1	Pga^- Tc^r (pLS20, pBC16)
3335 UM4 tr537B-15	Pga^- Tc^r (pLS20, pBC16)
3335 UM4 tr537B-16	Pga^- Tc^r $pLS20^-$ (pBC16)

TABLE 1. (continued)

3335 UM22	UM4 tr537B-16 pBC16$^-$ at 42°, no plasmids
3335 UM23	Ade$^-$ of UM22 by UV, no plasmids
3335 UM24	Str^r of UM23 by UV, no plasmids
3335 UM25	Rif^r rfm-7 of UM22 by UV, no plasmids
B. licheniformis	
9945A	Prototrophic, Pga$^+$ [C. Thorne]
9945A UM109	Arg$^-$ Pga$^-$ Str^r [C. Thorne]
11946	Prototrophic, Pga$^+$ [ATCC]
11946 UM13	Str^r of 11946 [C. Thorne]

Abbreviations: Ade, adenine; Ant, anthranilic acid; Arg, arginine; C, cured; Cap, synthesis of capsule; Cap^{+c}, CO_2-dependent capsule synthesis; Cap^{+a}, synthesis of capsules in air; Cry, synthesis of parasporal crystals; Ind, indole; Leu, leucine; Osp, oligosporogenous; Pga, synthesis of polyglutamic acid; pyrA, mutation conferring requirement for arginine plus uracil); Rif^r, phenotype for rifampicin resistance; rfm genotype for rifampicin resistance; Str, streptomycin; Tc^r, pBC16-encoded tetracycline resistance; Tet, tetracycline; td, transduction; tf, transformation; Tox, synthesis of toxin; Ura, uracil; ATCC, American Type Culture Collection; Microbiological Research Establishment, Porton, England; NRRL, Northern Regional Research Laboratory, Department of Agriculture, Peoria, Illinois. In the strain designations, tr denotes a transcipient strain and td denotes a transductant obtained by CP-51-mediated transduction. Names or abbreviations in brackets represent sources of particular strains.

TABLE 2. Transfer of pXO2 by CP-51-mediated transduction[a]

Donor	Recipient	Transductant
B. anthracis 6602 (pXO2) Cap^{+c}	B. anthracis UM23C1 Cap^{-}	B. anthracis UM23C1td10 (pXO2) Cap^{+c}
B. anthracis 6602 (pXO2) Cap^{+c}	B. cereus 569 UM20-1 Cap^{-}	B. cereus 569 UM20-1td10 (pXO2) Cap^{+c}
B. anthracis 6602 (pXO2) Cap^{+c}	B. anthracis M UM4 Cap^{-}	B. anthracis M UM15 (pXO2) Cap^{+c}

[a] The generalized transducing phage CP-51 was used to transduce pXO2 into B. cereus 569 UM20-1 and $pXO2^{-}$ strains of B. anthracis. Phage CP-54 lyses noncapsulated cells, but does not adsorb to capsulated cells. Thus, its application to transduction plates allowed selection of Cap^{+} transductants. All transduction plates were incubated in 20% CO_2. Cap^{+} isolates were subsequently tested for the ability to produce capsule in air.

TABLE 3. Transfer of pXO2 by mating

Donor	Recipient	Transcipient
B. cereus 569 UM20-1 tr60G-10[a] (pXO12::pXO2, pBC16) Cap^{+c} Cry^{+} Tc^{r} Ant^{-}	*B. anthracis* 6602 R4 C1 Cap^{-} Cry^{-} Tet^{s} Ind^{+}	*B. anthracis* 6602 R4 C1 tr16K-1 (pXO12::pXO2, pBC16) Cap^{+c} Cry^{+} Tc^{r} Ind^{+}
B. cereus 569 UM20-1 tr60G-10 (pXO12::pXO2, pBC16) Cap^{+c} Cry^{+} Tc^{r} Ant^{-}	*B. anthracis* 6602 R5 C1 Cap^{-} Cry^{-} Tet^{s} Ind^{+}	*B. anthracis* 6502 R5 C1 tr17K-1) (pXO12::pXO2, pBC16) Cap^{+c} Cry^{+} Tc^{r} Ind^{+}
B. anthracis M UM2 tr24K-5[b] (pXO14, pXO2.1, pBC16) Cap^{+a} Tc^{r} Rif^{s}	*B. anthracis* UM23 C1-2 Cap^{-} Tet^{s} Rif^{r}	*B. anthracis* UM23C1-2 tr30K-5 (pXO14, pXO2.1Δ1, pBC16) Cap^{+a} Tc^{r} Rif^{r}

[a] *B. cereus* 569 UM20-1 tr60G-10 carries a recombinant plasmid, designated pXO12::pXO2, which contains the fertility and crystal genes of the *B. thuringiensis* plasmid pXO12 and the capsule genes of pXO2 from *B. anthracis* 6602. This donor was mated with cured derivatives of 6602 R4 and 6602 R5, and selection was for transfer of the Tc^{r} plasmid pBC16. Tc^{r} *B. anthracis* transcipients were screened by phase microscopy for parasporal crystals and subsequently examined for the ability to produce capsules.

[b] *B. anthracis* M UM2 tr24K-5 was isolated from mating *B. thuringiensis* 4059 td1 with *B. anthracis* M UM2. This Spo^{-} transcipient originally carried pXO14, pXO2.1, and pBC16. When tr24K-5 was mated with *B. anthracis* UM23 C1-2, Cap^{+} transcipients aquired altered forms of pXO14 and pXO2.1. After serial transfer on NBY slants, tr24K-5 was found to be $pXO14^{-}$ and to carry an altered form of pXO2.1.

TABLE 4. Isolation of Cap+[a] B. anthracis colonies by phage CP-54 selection

Strain	No. of Mucoid Colonies Plate 1[a]	Plate 2[b]
6602	2	0
6602 R4	1	1
6602 R5	0	0
4229	0	0
M UM22	8	2
M UM2	TNC[c]	TNC
UM23 C1-2 tr30K-4	TNC	TNC

[a] CP-54 was spread onto lawn of cells.

[b] Cells were replica plated onto lawn of CP-54.

[c] TNC, too numerous to count.

TABLE 5. Test of various Bacillus strains for the ability to transfer pBC16 to B. subtilis

Donor	Recipient	Transcipients per membrane
B. thuringiensis 4042A UM8 td2 (pXO11, pXO12, pBC16)	B. subtilis 168	0
B. thuringiensis 4042A UM8-13 td1A (pXO11, pBC16)	B. subtilis 168	0
B. anthracis UM23 C2 tr237-10(pXO12, pBC16)	B. subtilis 168	2.4×10^4
B. cereus 569 UM20-1 tr251-1(pXO12, pBC16)	B. subtilis 168 Rif^r	0
B. cereus 569 UM20-1 tr2B-4(pXO11, pBC16)	B. subtilis 168 Rif^r	0

Tc^r transcipients were selected on Min 1C agar supplemented with 25 µg of tetracycline and 40 µg of tryptophan per ml. When the Rif^r mutant was the recipient, rifampicin (5 µg per ml) was added to the selective plates.

TABLE 6. Yields of tetracycline-resistant B. subtilis transcipients after various periods of incubating donor and recipient together

Mating mixture	Hours of Incubation	Transcipients per membrane
B. anthracis UM23 C1 tr237-10 (pXO12, pBC16)	0	0
plus	2	3.6×10^2
B. subtilis 168 *trpC2*	4	8.8×10^3
	6	1.1×10^4
	20	2.8×10^4

TABLE 7. Efficiency of pXO11, pXO12, pXO13, pXO14, and pXO16 donors in transferring pBC16 to B. subtilis

B. anthracis Donor	*B. subtilis* Recipient	Tc^r transcipients per membrane
Weybridge A UM23 C2 tr96B-3(pXO11, pBC16)	1A436 r^-m^-	0
	1A510 r^-m^-	0
Weybridge A UM23-C2 tr237-10(pXO12, pBC16)	1A436 r^-m^-	1.0×10^4
	1A510 r^-m^-	7.5×10^2
Weybridge A UM23 C1-1 tr359A-1(pXO1 , pBC16)	1A436 r^-m^-	5.7×10^4
	1A510 r^-m^-	1.1×10^4
Weybridge A UM23 C1-1 tr338A-1(pXO14, pBC16)	1A436 r^-m^-	2.0×10^2
	1A510 r^-m^-	2.5×10^3
Weybridge A UM23 C1-1 tr336A-4(pXO16, pBC16)	1A436 r^-m^-	1.5×10^2
	1A510 r^-m^-	9.0×10^1

TABLE 8. Transfer of pBC16 from B. subtilis (natto) 3335 UM8 to B. anthracis UM44-1 C9

Age of Cultures[a]	Donor cells per ml	Recipient cells per ml	Tc^r transcipients per ml	Frequency[b]
2 h	4.6×10^6	2.4×10^7	50	1.1×10^{-5}
3 h	5.5×10^6	6.9×10^7	90	1.6×10^{-5}
4 h	3.9×10^7	1.2×10^8	330	8.5×10^{-6}
5 h	4.7×10^7	1.2×10^8	890	1.9×10^{-5}

[a] Transfers (5% v/v) from 16-h BHI broth cultures were incubated for 2, 3, 4, or 5 h before equal volumes of donor and recipient cells were mixed and spread on DA membranes.

[b] Frequency was calculated as No. of transcipients/No. of donors.

TABLE 9. Test of some Bacillus species as recipients of pBC16 in matings with B. subtilis (natto) 3335 UM8[a]

Recipient strain	Donor cells per ml	Recipient cells per ml	Tc^r transcipients No. per ml	Frequency
B. anthracis UM44-1 C9	4.7×10^7	1.2×10^8	8.9×10^2	1.9×10^{-6}
B. anthracis UM44-1 C9	1.2×10^7	1.5×10^8	2.5×10^2	2.1×10^{-5}
B. cereus 569 UM20-1	5.6×10^6	5.3×10^8	5.0×10^3	1.0×10^{-3}
B. thuringiensis 4042A UM2-1	3.6×10^6	4.9×10^8	1.4×10^2	3.9×10^{-5}
B. megaterium PV229	1.5×10^7	2.5×10^8	3.5×10^1	2.3×10^{-6}
B. subtilis IA510	1.1×10^7	1.0×10^8	2.8×10^2	2.5×10^{-5}
B. subtilis PY143	4.5×10^7	1.1×10^7	8.0×10^1	1.8×10^{-6}
B. subtilis W23	5.0×10^4	1.9×10^8	5.6×10^2	1.1×10^{-2}

[a] Mating mixtures were plated on DA membranes on LG agar. After 5 h at 30C, membranes were transferred to LG agar containing tetracycline (25 µg/ml) and either streptomycin (200 µg/ml) or chloramphenical (15 µg/ml). Colonies were scored after 24 to 48 h. Membranes that were spread with either donor or recipient cells alone, yielded no Tc^r colonies.

TABLE 10. Restriction fragments of pXO2 generated by EcoRI and HaeIII

EcoR1			HaeIII	
Fragment	Size (kb)		Fragment	Size (kb)
1	9.6	9.6	1	>21
2	7.55	7.7	2	4.7
3	7.1	7.25	3	4.25
4	6.2	6.05	4	4.1
5	6.15	6.0	5	3.95
6	5.75	5.6	6	3.5
7	4.9	4.85	7	2.8
8	4.85	4.8	8	2.45
9	4.25	4.25	9	1.95
10	4.15	4.1	10	1.45
11	3.15	3.2	11	.85
12	2.9	2.85	12	.75
13	2.75	2.7		
14	2.05	2.1		
15	2.0	2.05		
16	1.8	1.85		
17	1.78	1.75		
18	1.75	1.65		
19	1.6	1.6		
20	1.5	1.55		
21	1.4	1.4		
22	.9	.95		
23	.8	.85		
24	.75	.8		
Total	85.63	85.5		

TABLE 11. Test for entry exclusion in matings between strains carrying different fertility plasmids[a]

Part A. Plasmid present in Weybridge UM44-1 To^r donor	To^r transcipients per ml with Weybridge A UM23 recipient carrying plasmid:					
	none	pXO11	pXO12	pXO13	pXO14	pXO16
pXO12	4.6×10^4		2.1×10^3	5.1×10^5	7.3×10^4	2.1×10^5
pXO13	1.6×10^4	4.3×10^4	1.0×10^5	7.4×10^2	4.5×10^4	9.3×10^4
pXO14	6.9×10^4	1.1×10^5	7.8×10^4	2.5×10^5	1.1×10^3	2.4×10^5
pXO16	4.2×10^4	1.0×10^5	1.4×10^5	7.5×10^4	6.3×10^4	1.5×10^3

Part B. Donor To^r strain	To^r transcipients per ml obtained with a B. cereus 569 UM20-1 recipient containing the plasmid				
	none	pXO11	pXO12	pXO14	pXO15
B. cereus 569 UM47 tr292A-11(pXO13)	4.1×10^2	NT[b]	NT	NT	4.2×10^2
B. cereus 569 UM47 tr320A-3(pXO14)	1.3×10^3	NT	NT	NT	1.9×10^3
B. cereus 569 UM34 tr163A-1(pXO16)	1.0×10^5	NT	NT	NT	1.4×10^5
B. thuringiensis YAL UM1(pXO15)	9.8×10^4	6.2×10^4	6.5×10^4	1.4×10^5	8.0×10^1

[a] Donor strains in the matings in (A) were Ind^- auxotrophs and the recipients were Ura^- auxotrophs. Transcipients were scored by plating the mating mixtures on MinIC agar containing 5 µg of tetracycline and 40 µg of uracil per ml. To^r transcipients in (B) were determined by plating mating mixtures on L agar supplemented with 25 µg of tetracycline and 200 µg of streptomycin per ml.
[b] NT, Not tested

TABLE 12. Plasmids present in transcipients derived from matings between strains containing different fertility plasmids

Fertility Plasmid in donor strain	Fertility plasmid in recipient strain	Fertility plasmids found in transcipients analyzed[a]
pXO11	pXO13	14/16 pXO11 + pXO13; 2/16 pXO13
"	pXO16	7/8 pXO11 + pXO16; 1/8 pXO11
pXO12	pXO14	2/16 pXO12 + pXO14; 1/16 pXO12; 3/16 pXO14
"	pXO16	10/14 pXO12 + pXO16; 3/14 pXO12; 1/14 pXO16
pXO13	pXO13	3/3 pXO13
"	pXO14	7/18 pXO13; 11/18 pXO14
"	pXO15	4/4 pXO13 + pXO15
"	pXO16	5/8 pXO13 + pXO16; 3/8 pXO16
pXO14	pXO13	14/17 pXO14; 3/17 pXO13
"	pXO14	5/5 pXO14
"	pXO15	5/5 pXO14 + pXO15
"	pXO16	3/5 pXO14 + pXO16; 2/5 pXO16
pXO16	pXO15	8/8 pXO16 + pXO15

[a] The values represent the number of transcipients containing particular fertility plasmids/the number analyzed for plasmid content.

TABLE 13. Restriction endonuclease cleavage sites in pXO11 and pXO12

Enzyme	Number of restriction fragments generated with	
	pXO12	pXO11
PstI	7	7
XbaI	ca. 21[a]	18
ClaI	15	14
KpnI	6	5
SalI	3	1
EcoRI	ca. 22[b]	14
HindIII	ca. 24	ca. 18
HaeIII	ca. 20	ca. 18
BamHI	1	1
BglI	1	1
SmaI	1	1

[a]Estimated number of fragments. XbaI digestions yielded variable cleavage patterns for pXO12. Low molecular weight fragments were barely visible in 0.7% agarose gels under UV illumination or in 35-mm photographs.
[b]Fragment numbers represent estimated number of fragments. EcoRI, HindIII, and HaeIII digests yielded a large number of fragments of which the low molecular weight ones were very faint in 0.7% agarose gels and therefore difficult to quantitate.

TABLE 14. Data for size estimation of plasmids pXO11 and pXO12

Number and size of restriction fragments generated upon digestion with[a]									
	PstI			ClaI				XbaI	
	pXO12		pXO11		pXO12		pXO11		pXO11
1	70.0[b]	1	47.8	1	59.8	1	30.0	1	16.6[b]
2	13.0	2	12.1	2	13.4	2	20.7	2	11.4
3	10.0	3	11.5	3	10.5	3	10.75	3	10.0
4	5.9	4	6.5	4	4.75	4	3.9	4	9.3
5	4.5	5	3.2	5	3.2	5	3.05	5	7.9
6	3.1	6	3.1	6	3.0	6	2.75	6	4.05
7	1.8	7	2.2	7	2.6	7	2.5	7	3.3
				8	2.5	8	2.4	8	3.1
				9	2.4	9	2.0	9	2.9
				10	2.2	10	2.0	10	2.6
				11	1.8	11	1.7	11	2.5
				12	1.7	12	1.25	12	2.3
				13	1.6	13	.6	13	1.9
				14	1.55	14	.4	14	1.75
				15	1.45			15	1.65
								16	.9
								17	.7
								18	.35
Sums:									
108.3 kb		86.4 kb		112.5 kb		84.0 kb		83.2 kb	

[a] pXO11 and pXO12 fragments generated from PstI and ClaI digestion were sized using 0.3% and 0.7% agarose gels for large (10 to 70 kb) and small (1.0 to 7.0 kb) fragments, respectively. pXO11 fragments generated from XbaI digestion were sized using 0.7% agarose gels.

[b] Numbers were extrapolated from standard curve.

TABLE 15. Characteristics of Tra$^+$ deletion derivatives of pXO12

Deletion plasmid	Relevant characteristics[a]	Origin
Class I (ca. 90 to 100 kilobases)		
pXO12Δ223	Cry$^+$ Tra$^+$ (LF)	42°C
pXO12Δ379	Cry$^+$ Tra$^+$ (LF)	Mating[b]
pXO12Δ388	Cry$^+$ Tra$^+$ (NF)	Mating
Class II (ca. 84 kilobases)		
pXO12Δ44	Cry$^-$ Tra$^+$ (LF)	Mating
pXO12Δ237	Cry$^-$ Tra$^+$ (LF)	42°C
pXO12Δ373	Cry$^-$ Tra$^+$ (LF)	Mating
pXO12Δ410	Cry$^-$ Tra$^+$ (NF)	Mating

[a]Abbreviations: Tra$^+$ (LF), lower frequency of pBC16 transfer than with wild-type pXO12; Tra$^+$ (NF), same frequency of pBC16 transfer as with wild-type pXO12.
[b]Mating, isolation of pXO12 deletion derivatives from transcipients following mating with wild-type pXO12.

TABLE 16. Deletion derivatives of plasmids pXO11 and pXO12

Plasmid	Size[a] (kb)	Characteristics[b]	Origin
Wild-type pXO12	112.5	Cry^+ Tra^+ (HF)	4042A M8 td2
Deletion mutants of pXO12			
pXO12Δ203	90 to 100	Cry^+ Tra^-	42°C
pXO12Δ43	90 to 100	Cry^+ Tra^-	42°C
pXO12Δ47	84	Cry^+ Tra^-	42°C
Wild-type pXO11	84	Cry^- Tra^+ (NF)	4042A M8-13 td1-A
Deletion mutant of pXO11			
pXO11Δ418	71	Cry^- Tra^+ (HF)	Mating[c]

[a]Plasmids were sized following electrophoresis in 0.6% agarose gels.

[b]Abbreviations: Tra^+ (NF), normal frequency of pBC16 transfer; Tra^+ (HF), higher than normal frequency of pBC16 transfer.

[c]Mating, isolation of deletion derivative from transcipient derived from a mating in which the donor carried a wild-type fertiliy plasmid.

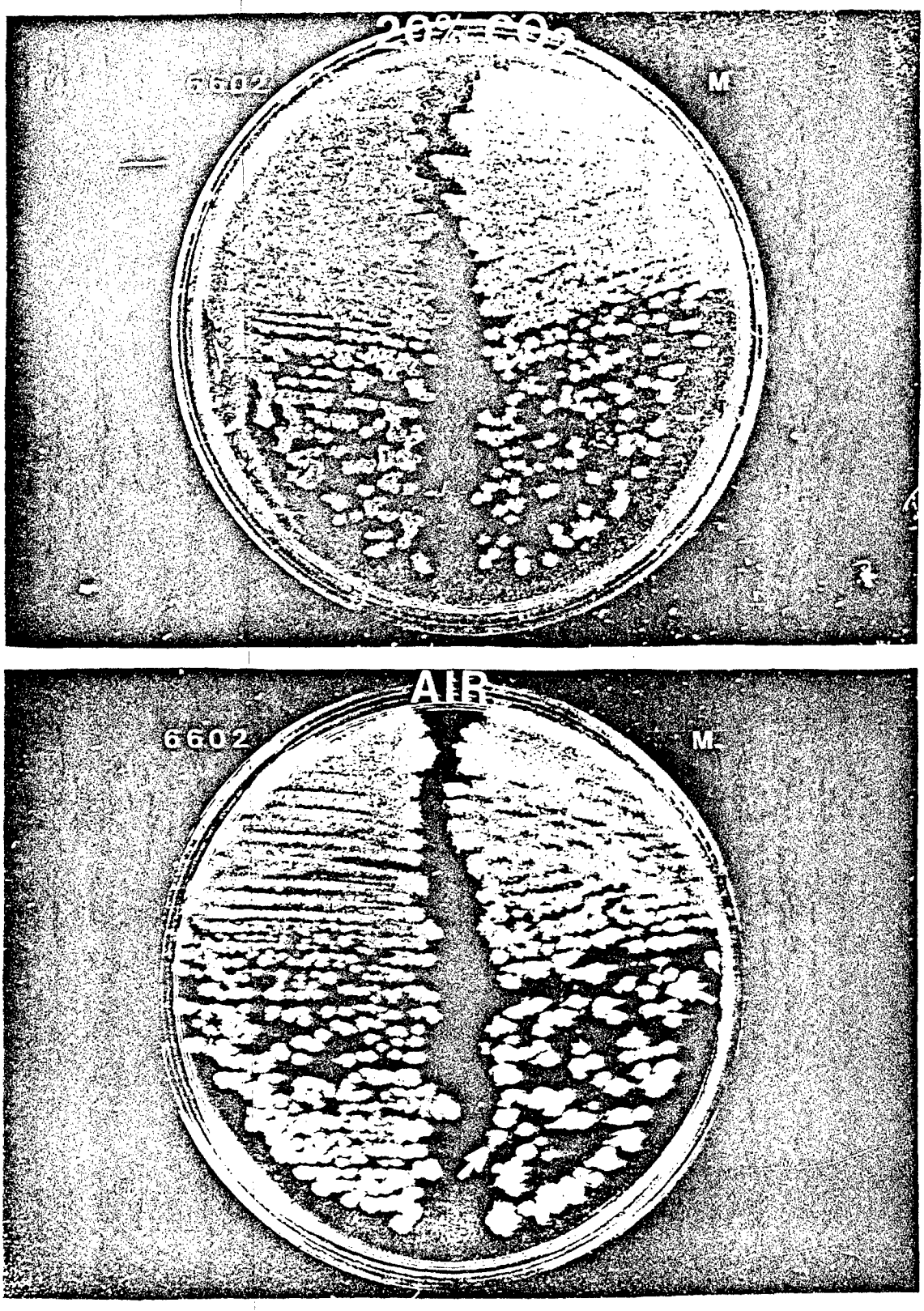

FIG. 1. Colonies of B. anthracis 6602 and M grown on PA agar in air and on $PACO_3$ agar in 20% CO_2. Arrows indicate spontaneous rough variants of M.

PUBLICATIONS

The following abstracts were published during this reporting period:

Reddy, A., L. Battisti, and C. B. Thorne. Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985.

Heemskerk, D., L. Battisti, and C. B. Thorne. Transfer of the Bacillus thuringiensis fertility plasmid pXO12 from Bacillus anthracis to Bacillus subtilis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985.

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